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READY TO PULL

FORCE TRANSMISSION DURING VASCULAR DEVELOPMENT

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Stockholm 2020

Cover: Vascular Universe
Description: Intriguing blood vessel network in murine urinary bladder.

Original image capture: Yuanyuan Zhang
Artistic modification: Yu-Hsuan Hsu

Published by Karolinska Institutet.
Printed by US-AB Stockholm

ISBN 978-91-7831-628-1

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Force Transmission during Vascular Development

THESIS FOR DOCTORAL DEGREE (Ph.D.)

Public defence at Cancer Centrum Karolinska (CCK) Lecture hall, R8:00, Karolinska University Hospital (Solna), Stockholm, Sweden.

Friday, January 17th 2020, at 09:00 am.

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To science

KEY WORDS

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ABSTRACT

The transmission of mechanical forces controls a multitude of cellular processes such as migration, the formation of new organs, muscle contraction, and resistance to the continuous stress that blood flow exerts on the blood vessel wall.

These forces are sensed and replayed at the sites of cellular adhesion to the basal membrane as well as cell-cell junctions, where protein complexes connect to the cellular cytoskeleton to relay force into the cell. During vascular development, endothelial cells (ECs) continuously sense and respond to mechanical cues from the microenvironment to form a functional and hierarchical vessel network. Uncovering the underlying mechanisms, by which the mechanical tension is generated, sensed, and relayed into the cell is important in advancing our understanding of the pathogenesis of vascular disease. In this thesis, evidence is presented for a role of the Angiomotin (Amot) scaffold protein family as essential mediators in the vascular endothelium.

As stated in **Paper I**, deletion of Amot in the endothelium inhibits the expansion of the physiological and pathological vascular network. Furthermore, we provide evidence that Amot is a novel component of the integrin adhesome important for linking fibronectin (Fn) in the extra-cellular matrix (ECM) to the intercellular actin filaments. Fn is a major component in the ECM, which is sensitive to mechanical forces resulting in extensive molecular elongation. In **Paper II**, the force-sensitive "integrin switch" of Fn-binding sites is experimentally confirmed, and an engineered scFv named H5 is developed, which specifically binds to that force-induced conformational change of Fn. We provide further experimental evidence both *in vitro* and *ex vivo* for the H5 ability to detect and target the early molecular signatures of cell contractile forces *in vivo*.

Transmission of mechanical force occurs not only via cell-matrix adhesions but also cell-cell junctions. As described in **Paper III**, AmotL1 forms a complex with N-cadherin presented on both ECs and pericytes. Exploiting endothelial-specific knockout mice, we demonstrate that AmotL1 is essential for normal establishment of vascular networks in the post-natal mouse retina and in a transgenic breast cancer model. In **Paper IV**, we indicate that AmotL2 connects junctional VE-cadherin and radial actin filaments to the LINC complex in the nuclear membrane. Deletion of AmotL2 impairs the formation of radial actin filaments and the flow-induced alignment of aortic ECs and affects nuclear shape and positioning. Moreover, the absence of AmotL2 in ECs provokes a pro-inflammatory response and abdominal aortic aneurysms in the aortae of adult mice that are consuming a normal diet.

Overall, these findings provide a conceptual framework regarding force mechanotransduction via cell-matrix adhesions and cell-cell junctions that are associated with vascular physiology and relevant diseases.

LIST OF SCIENTIFIC PAPERS

I. The Amot/Integrin protein complex transmits mechanical forces required for vascular expansion.
Yuanyuan Zhang, Sumako Kameishi-Kondo, Yujuan Zheng, Giuseppina Barutello, Yumeng Zhang, Nicholas P. Tobin, John Nicosia, Katharina Hennig, Kungchun Chiu, Martial Balland, Thomas H. Barker, Federica Cavallo, and Lars Holmgren#.
Manuscript.

II. Detection of an integrin-binding mechanoswitch within fibronectin during tissue formation and fibrosis.
Lizhi Cao*, John Nicosia*, Jacqueline Larouche, Yuanyuan Zhang, Haylee Bachman, Ashley C. Brown, Lars Holmgren, and Thomas H. Barker#.
ACS Nano. 2017 Jul 25;11(7):7110-7117.

III. Angiomotin like-1 is a novel component of the N-cadherin complex affecting endothelial/pericyte interaction in normal and tumor angiogenesis.
Yujuan Zheng, Yuanyuan Zhang, Giuseppina Barutello, Kungchun Chiu, Maddalena Arigoni, Costanza Giampietro, Federica Cavallo & Lars Holmgren#.
Scientific Reports. 2016 Jul 28;6:30622.

IV. The VE-cadherin/AmotL2 mechanosensory pathway suppresses aortic inflammation and the formation of abdominal aortic aneurysms.
Yuanyuan Zhang, Evelyn Hutterer, Sara Hultin, Otto Bergman, Maria J. Forteza, Daniel Ketelhuth, Anders Franco-Cereceda, Per Eriksson, and Lars Holmgren#.
Manuscript.

OTHER PAPERS NOT INCLUDED IN THIS THESIS

I. Arsenic primes human bone marrow CD34+ cells for erythroid differentiation.
Yuanyuan Zhang, Shasha Wang, Chunyan Chen, Xiao Wu, Qunye Zhang, and Fan Jiang#.
Bioinorganic Chemistry and Applications. 2015; 2015: 751013.

II. The E-cadherin/AmotL2 complex organizes actin filaments required for epithelial hexagonal packing and blastocyst hatching
Sebastian Hildebrand, Sara Hultin, Aravindh Subramani, Sophie Petropoulos, Yuanyuan Zhang, Xiaofang Cao, John Mpindi, Olli Kalloniemi, Staffan Johansson, Arindam Majumdar, Fredrik Lanner, and Lars Holmgren#.
Scientific Reports. 2017 Aug 25;7(1):9540.

**: Equal contribution; #: Corresponding authors*

CONTENTS

LIST OF ABBREVIATIONS

AAA	Abdominal aortic aneurysm
AJ	Adhesion junction
Amot	Angiomotin
AmotL1	Angiomotin like 1
AmotL2	Angiomotin like 2
CCL2/5	C-C motif chemokine ligand 2/5
cDNA	complementary DNA
Cre	Cre recombinase
CTGF	Connective tissue growth factor
CVD	Cardiovascular disease
CXCL10	C-X-C motif chemokine 10
Cyr61	Cysteine-rich angiogenic inducer 61
DNA	Deoxyribonucleic acid
E-cadherin (Cdh1)	Epithelial cadherin
EC	Endothelial cell
ECM	Extracellular matrix
F-actin	Filamentous actin
FA	Focal adhesion
FAK	Focal adhesion kinase
Fn	Fibronectin
G-actin	Globular actin
GAP	GTPase activating proteins
HAoEC	Human aortic endothelial cell
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HUVEC	Human umbilical vein endothelial cell
IB4	Isolectin B4
ICAM-1	Intercellular adhesion molecule 1
IL6	Interleukin 6
ITG	Integrin
JAM	Junctional adhesion molecule

KD/KO	Knockdown/Knockout
LATS	Large Tumor Suppressor kinase
LINC	Linker of Nucleoskeleton and Cytoskeleton
MDCK	Madine darby canine kidney
mRNA	messenger RNA
N-cadherin (Cdh2)	Neural cadherin
NF2	Neurofibromatosis type 2
OB-cadherin (Cdh11)	Osteoblast cadherin
P-(day number)	Postnatal day
PECAM	Platelet endothelial cell adhesion molecule
Rac1	Ras-related C3 botulinum toxin substrate 1
RhoA	Ras homolog family member A
ROCK	Rho associated coiled-coil containing protein kinase
ROSA26	Rosa26 locus reporter
ScFv	Single-chain variable fragment
shRNA	short hairpin RNA
siRNA	small interfering RNA
TAZ	Transcriptional co-activator with PDZ-binding motif
TNF	Tumor necrosis factor
VCAM-1	Vascular cell adhesion protein 1
VE-cadherin (Cdh5)	Vascular endothelial cadherin
VEGF(R)	Vascular endothelial growth factor (receptor)
vSMC	vascular smooth muscle cell
WT	Wild type
YAP	Yes-associated protein
YFP	Yellow fluorescent protein
ZO-1	Zonula adherens-1

1 INTRODUCTION

Force is a general concept that describes the push or pull on an object resulting from its interaction with another object. When the interaction ceases, the force is no longer applied. As a vector, force has both magnitude and direction, and it can thus be measured and quantified along a certain direction in a specific time point. Force is classified into two broad categories: action-at-a-distance force and contact force. As its name suggests, contact force, unlike action-at-a-distance forces, such as gravitational and electrical force, is exerted when two objects come into a direct physical contact. The force discussed in this thesis is mainly tension (tensional force), one of the most common contact forces.

As illustrated in ***Figure 1***, the tension force is the force transmitted through a rope or similar string when it is pulled taught by force acting from opposite ends. The length of the rope determines the significant distance over which the force can be transferred.

Figure 1. Ready to pull. *The tension force is generated by two athletes and transmitted axially through the rope. The statue "Kamp" (Struggle) was exhibited at the National Sports Museum in Stockholm. Permission for adaptation was obtained from Swedish artist, Assa Kauppi, and the Tore A Jonasson foundation.*

1.1 Mechanobiology

In the context of the field of biology, mechanical force influences the morphology, mobility and functionality of virtually every cell, tissue and organ in our bodies. Once the mechanical properties of tissues are altered, certain human diseases may occur. In recent decades, an increasing number of studies have aimed to characterize the process of force sensing, transmission and transduction into bio-signals, to reveal molecules and mechanisms of coordinating cell internal structure and external mechanical inputs and so on. The subject of mechanobiology therefore emerged at the interface of physics and biology, and it encompasses novel biotechnologies and experimental approaches, as well as cell and development biology.

As early as 1943, Holtfreter, an embryologist of Entwicklungsmechnik (developmental mechanics) movement, had articulated embryo morphogenesis as a physical, mechanical issue: that is, cells generate force to remodel the embryo (Holtfreter, 1943). Shortly thereafter, he published two hypotheses explaining the movement of embryonic tissues. One of these is the concept of mechanically integrated cell migrating behavior (Holtfreter, 1944; Keller, 2012). In the 1980s, Donald Ingber and colleagues reported that the extracellular

matrix (ECM) surrounding the cells has an impact on cell function and DNA synthesis (Sauer, 1987). Due to limitations in technique, the mechanisms were not well explained. This holistic mechanical view was overwhelmed by reductionist studies of molecular biology at the genetic level. However with the rapid development of approaches with which to manipulate and measure force, mechanical forces have gradually returned to the spotlight of modern research in various areas, such as embryogenesis, tissue development, stem cells and cancer progression (Piccolo, 2013; Schwartz, 2010a; Butcher, Alliston and Weaver, 2009; Fredberg *et al.*, 2009; Wozniak and Chen, 2009; Bissell and Hines, 2011). It appears that the biological effect of forces are not limited to physical activity from heart pumping to single cell migration, but can also regulate a wide variety of processes, such as cell proliferation and tissue homeostasis.

To explore how mechanical forces affect the cells, we need to consider that most cells in our bodies are not solitaries, but in direct physical contact with the environment, including the ECM, or other neighboring cells, or, often both. It is a starting point to recognize and understand the cellular structural basis on which forces are generated, transmitted and transduced into biochemical signals.

A simplified schematic in ***Figure 2*** presents three key contributors to cellular mechano-sensing and -adaption: integrin mediated focal adhesion (FA) between the cell and ECM, tissue-specific cadherin based adhesion junction (AJ) and internal contractile machinery formed by actin-myosin network. Both the cell surface complexes, that is, FAs and AJs, link associated receptors to actin cytoskeleton (Sastry and Burridge, 2000; Gumbiner, 2005). Of course, the constructional and functional situation in reality is much more sophisticated than the simplified diagram below presents.

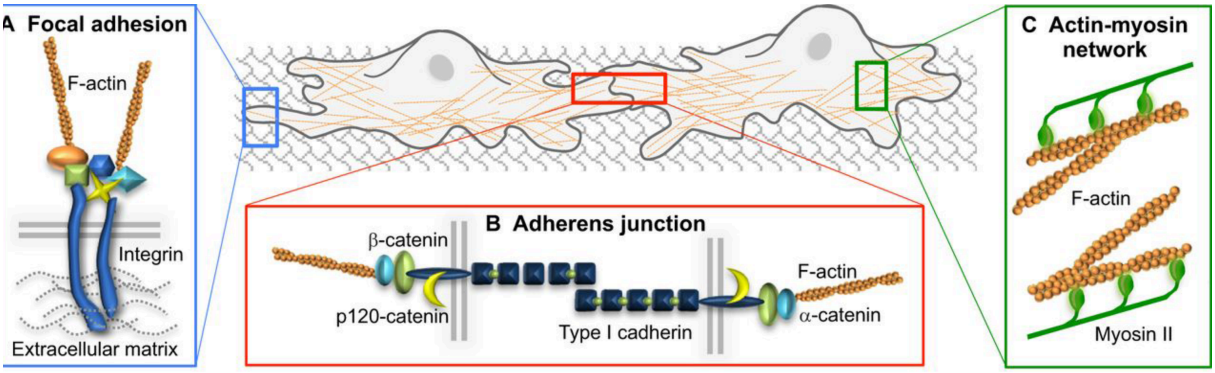


Figure 2. Mechanical landscape of the cell. *(A) Integrin-based focal adhesions. (B) Cadherin-dependent adhesive junctions. (C) Contractile actin–myosin network. Adapted from (Mui, Chen and Assoian, 2016). Reprinted with permission from the publisher.*

1.1.1 Cell-Matrix Adhesion

Cell attachment to the surrounding ECM components with specialized structures is termed "cell-matrix adhesion."

1.1.1.1 ECM components

The ECM forms the complex niche of structural elements that surround cells *in vivo*, and is commonly known as connective tissue in animals. It provides structural and biochemical support for the cell anchorage required for cell morphogenesis, mobility and differentiation. In return, the cells that sense the environmental stimuli are also capable of deforming and remodeling ECM in response.

Fundamentally, the ECM consists of water, polysaccharides and approximately 300 proteins, and the composition and properties of ECM is tissue-specific and also strikingly heterogeneous. Proteoglycans (PGs) and fibrous proteins are the two main classes of macromolecules comprising ECM. Proteoglycans form a hydrated gel-like network filling the interstitial space and regulating tissue properties and cell binding to ECM (Schaefer and Schaefer, 2010). Collagens, presenting as fibrous protein, are the most abundant protein in ECM, and give structural support to surrounding cells. Elastin, by contrast, gives elasticity to tissues as bungee that allows stretching when needed. Tissues such as blood vessels and skin contain high amounts of elastin in order to absorb mechanical stress. For instance, the degradation of elastin in aortic wall is one of the most common predisposing factors for aortic dissection, since the stiffer wall is not able to sustain the pulsatile blood flow (Tsamis, Krawiec and Vorp, 2013). Collagen and elastin are closely associated and the delicate balance of their stoichiometry determines ECM stiffness and elasticity (Bonnans, Chou and Werb, 2014).

Fibronectin (Fn), another large fibrous protein (450 kD), not only is involved in organizing interstitial ECM, but also plays a key role in mediating cell adhesions. The protein consists of homologous repeating structural motifs termed type I, II, and III modules, which contains binding sites for cells, collagens, and other ECM partners (***Figure 3***). The motif in FnIII binding cells through integrins is a tripeptide called Arg-Gly-Asp (RGD).

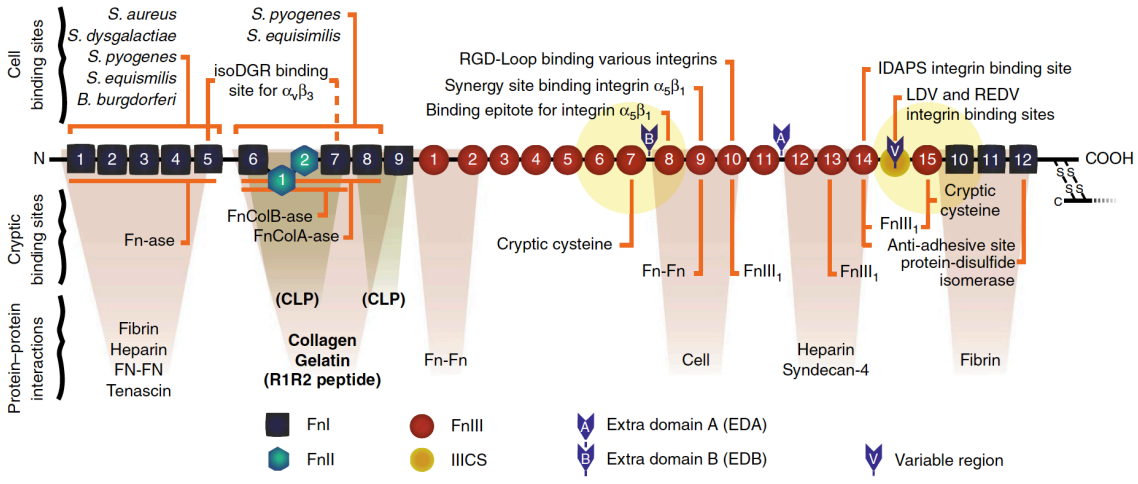


Figure 3. Fn's major binding sites. *The primary structure of Fn, displayed with binding sites to different proteins. Adapted from open-access article (Kubow et al., 2015), which was the modified version of Figure 1 in (Antia et al., 2008). Reprinted with permission from the publisher.*

Both *in vitro* and *in vivo* evidence has supported that Fn exhibits specialized structural states subjected to cellular forces within ECM (Chandler *et al.*, 2011; Cao *et al.*, 2012). Cell-mediated Fn fibril assembly is tightly regulated by mechanical signals (Zhong *et al.*, 1998). The traction generated by cells is sufficient to extend the fibers at least 5-6 fold through multiple modules unfolding (Little *et al.*, 2008). These conformational changes can expose the cryptic sites, which remain hidden under circumstances characterized by low mechanical stress. Additionally, the exposure of cryptic sites in FnIII, for instance, is an activator and regulator for integrin binding and cell signaling affecting cytoskeleton formation and organization (Smith *et al.*, 2007). Conversely, a change in cytoskeleton contractility is able to alter the same FnIII cryptic sites. Manipulating cell contractility by inhibiting actomyosin contractility using cytochalasin D or Rho A inhibitor caused the similar remolding of FnIII in fibrils *in vitro* (Baneyx, Baugh and Vogel, 2002).

1.1.1.2 ECM stiffness

Because the specific composition of ECM is organ-dependent, different tissues display varying levels of stiffness, from the soft tissue of the lungs (≈200 Pascals, Pa in short) and breast (≈800 Pa) to that of muscle (≈12, 000 Pa) and bone (2-4 x 10^9 Pa) (Butcher, Alliston and Weaver, 2009). Collagens are the major contributors to the physical properties of a tissue due to its abundance and intricate glycosylation and crosslinks with other fibrous proteins, such as elastin and Fn (Erat *et al.*, 2013). However, the collagen matrix could not form in an Fn-deficient fibroblast cell culture without exogenous Fn (Velling *et al.*, 2002). In addition, defective Fn has contributed to stiffer ECM due to massive collagen deposition in a mouse model (Iwasaki *et al.*, 2016). These findings indicate that Fn is a critical regulator of ECM stiffness.

An uncontrolled increase in the cross-linking between Fn and collagen proteins can lead to more ECM rigidity, whereas too little communication results in ECM degradation. Both perturbations can cause a number of tissue behaviors to go awry, eventually leading to severe disease, such as fibrosis or cancer (Najafi, Farhood and Mortezaee, 2019). Changes in ECM stiffness can occur in very early stages. Therefore, early detection and reversal of abnormal ECM properties is important in the diagnosis and treatment of disease.

1.1.1.3 Integrin associated focal adhesion

How are mechanical cues transmitted into the cell?

Cells form force-sensitive integrin-containing protein complexes called focal adhesion (FA) at the interface between intracellular actin bundles and the extracellular substrate. Focal adhesion is distinguished from other adhesive structures for its response to tension as seen in ***Table 1***. Focal adhesion assembly and disassembly can be highly dynamic depending on the mechanical instruction from both sides of the cell membrane (Zamir *et al.*, 2000). Thus, FAs are generally considered to be the mechanosensory units that integrate multiple spatiotemporal cues and interpret into pathways controlling cellular decisions.

Term	Character
Focal adhesion	Large adhesion connected by F-actin stress fibers in response to tension
Focal complexes	Small adhesions in membrane protrusions of spreading and migrating cells
Fibrillar adhesion	Elongated adhesions associated with fibronectin matrix assembly
Nascent adhesion	Smallest adhesive structures merged at the edge of protruding membranes
Podosomes/Invadopodia	Proteolytically active adhesion found in osteoclasts, macrophages and certain cancer cells

Table 1. Terms regarding cell-matrix adhesions. *Summarized from (Sun, Lambacher and Fässler, 2014; Truong and Danen, 2009).*

The major bi-directional signaling receptors are integrins. In the 1980s, Hynes and colleagues first cloned a cell membrane protein, which was found to be the major adhesion receptor and subsequently named integrin (Tamkun *et al.*, 1986). Integrins function as heterodimeric glycoproteins with an α- and a β-subunit. So far 18 integrin α-subunit genes and 8 integrin β-subunit genes have been discovered in humans, generating 24 known binding pairs as integrin heterodimers (Takada, Ye and Simon, 2007). They exhibit distinct specificity in binding diverse matrix molecules, for instance, Fn, fibrinogen, collagen, laminin et cetera.

Integrin activity is modulated by changes in ligand affinity (low, intermediate and high), both spatially and temporally (Luo, Carman and Springer, 2007). With respect to structure, integrin activation can be recognized by the complete extension or separation of the cytoplasmic leg domains. The mechanical signals generated by integrins can modulate cell adhesion dynamics and influence both cell functions and ECM remodeling (Ginsberg, 2014). When the cells are stimulated to migrate in processes such as wound healing, angiogenesis, the cells frequently generate and deform the anchorages on ECM mediated by a specific profile of integrins (which integrins are involved and if the integrin is activated, for example). Truong's team has proposed an "integrin switch" theory in Fn-binding of α5β1- and αvβ3-integrin, in which a shifted integrin profile can dramatically affect the FA dynamic and cell motility (Truong and Danen, 2009).

1.1.1.4 Proteins associated with FA

Integrins recruits more than 180 proteins to FAs, including FA structural adaptors, scaffolding and catalytic adaptors et cetera. Classically, talin and vinculin are well-known mechanosensitive proteins (Rio *et al.*, 2009; Grashoff *et al.*, 2010), both of which are crucial for stabilizing FAs and engaging contractile actin filaments. Talin is a multi-domain protein that contains binding sites for the integrin cytoplasmic domain in the N-terminal as well as for F-actin binding sites in the C-terminal (Calderwood, Campbell and Critchley, 2013). In the relaxed state, talin's cryptic sites are not exposed for binding; however, cytoskeleton contraction stretches talin to unfold vinculin-binding sites. Activated vinculin stabilizes the open conformation of talin to enhance the mechanical connection between integrin and the actomyosin cytoskeleton (Yao *et al.*, 2014a). Additionally, paxillin have been demonstrated to primarily form bridges for FA proteins to physically attach to (Schaller, 2001); in another way, focal adhesion kinase (FAK) is able to amplify the signals transduced from FAs by

phosphorylation on cytoplasmic tail residues in response to integrin engagement and other stimuli (Deramaudt *et al.*, 2011).

1.1.2 Cell-Cell adhesion

Cells *in vivo* are not only attached to ECM, but also closely glued to adjacent cells via cell-cell contacts. Stable adhesion between cells contributes to the integrity of the tissue, while dynamic cell-cell adhesions provides molecular basis for development of multicellular tissue architecture and morphogenesis (Gumbiner, 1996). Besides providing physical attachment, adhesive structures also enable intercellular communications of mechanical, chemical, or electrical signals. So far there are several types of cell-cell adhesive junctions that have been reported to be sensitive and responsive to mechanical force:

1.1.2.1 Adhesion Junction (AJ)

An AJ is a stable cell anchoring that serves as an interconnected lateral bridge linking that contractile actin cytoskeleton of one cell to that of another. The core AJ components are the cadherin (named for "calcium dependent" adhesion), which bind cell together. Classical cadherins are cell-type specific; for example, E-cadherin (Cdh1) is found in epithelial tissue, VE-cadherin (Cdh5) in vascular endothelium and N-cadherin (Cdh2) in neurons and mesenchymal cells, such as pericytes and fibroblasts. Depending on contacts occurring between identical or different cell types, AJs can be described, respectively, as either homotypic or heterotypic. For example, endothelia form the luminal surface by VE-cadherin between cells, but form N-cadherin based contact with mural cells (e.g., pericyte).

Initial clues that AJs respond to mechanical force were derived from the observation that AJ size and strength can be altered by mechanical force (Liu *et al.*, 2010; Thomas *et al.*, 2013). More directly, using magnetic twisting cytometry to apply force to E-cadherin, cell adhesions were stiffened (le Duc *et al.*, 2010). With the knowledge of cadherins probing and transmitting mechanical force, extensive studies have focused on the cytoplasmic components that mediate the force to the cytoskeleton. β-catenin is one the most important linker proteins and has been identified to not only bind E-cadherin intercellular domains, but also to directly couple to α-catenin and vinculin, both of which link to the actin cytoskeleton (McCrea, Turck and Gumbiner, 1991; Jockusch and Isenberg, 1981).

1.1.2.2 Tight junction (TJ, also known as zonula occludens)

Tight junctions are intercellular junctional complex formed in epithelium and endothelium that make up the epithelial and endothelial diffusion barriers (Steed, Balda and Matter, 2010). The components of TJs are initially recruited to cadherin-based AJ and are thereafter relocated to the distinct apical side of the cells as junctions mature. Topologically, TJs are closely intermingled with AJs, especially strong in endothelium (Dejana, 2004). In addition to functions of maintaining cell polarity and preventing the leakage of ions and solutes, the relationship between TJ biology and mechanical force has begun to be noticed (Citi, 2019). The common integral membrane proteins include transmembrane proteins (claudin, occludin

and tricellulin) and single-span transmembrane proteins, such as junctional adhesion molecules (JAMs). Similar to AJs, the cytoplasmic scaffold proteins at TJs interact with cytoskeleton through the cytoplasmic scaffold proteins such as ZO (zonula occludens)-1, MAGI-1 (membrane-associated guanylate kinase inverted proteins) and polarity proteins PAR-3, PLLS-1 and PATJ (Zihni *et al.*, 2016; Fanning, Itallie and Anderson, 2012).

1.1.2.3 Desmosome (also known as macula adherens)

Desmosomes adopt a hyper-adhesive manner to resist mechanical stress as intercellular junctions. Instead of association via dynamic actin filaments, cadherin-like transmembrane and linker proteins form strong scaffolding poles with keratin intermediate filament, termed DIFC (desmosome-intermediate filament complex). This firm structure strengthens cell-cell binding, which is pivotal for tissue integrity, but with less ability to adjust transient and temporal mechanic cues (Garrod and Chidgey, 2008). Desmosome structures are commonly found in epithelia and cardiac muscle, but not in endothelium (Dejana, 2004).

1.1.2.4 Gap junction (GJ) and ion channel

Gap junctions are specialized intercellular connections between the cytoplasm of two cells, functioning as a regulated gate that selectively permits the direct passage of small molecules. These connexin-based channels are able to react with mechanical forces in vascular tissues and bones (Salameh and Dhein, 2013). For instance, the three-fold up-regulation of Cx43 (one of the connexin proteins), together with an enlarged GJ size, has been observed within four hours of cyclic mechanical stretch on cultured rat cardiomyocytes (Wang, Tseng and Chang, 2000). An ion channel is another type of spot-like structure between two attaching cells that permits the direct transportation of small ion molecules such as K^+ and Na^+, Notably, ion channels have also been reported as a mechanosensor of substrate rigidity through stress fibers and FAs (Kobayashi and Sokabe, 2010).

1.1.3 Cytoskeleton network

After the mechanical force comes through the surface-adhesion receptors, such as integrins and cadherins, the stress either dissipates quickly to the viscous cytoplasm or continues passing through channels with certain tensegrity. The solid network built to maintain cell structure is termed the cytoskeleton, and it is the fundamental structure inside cells to mediate the force transmission (Wang, Butler and Ingber, 1993). The cytoskeletal proteins can be divided into three main groups.

1.1.3.1 Intermediate filament (IF)

Averaging 10 nm in diameter, IFs are the stable and durable structure protecting the cell from external mechanical stress, anchoring organelles and composing nuclear laminas. As mentioned earlier, IFs are one of the components of DIFC (desmosome-intermediate filament complexes) at desmosomes connecting to neighboring cells. There is a high diversity of IF expression depending on cell type: Keratins are primarily expressed in epithelial cells, and desmin in muscle cells, for example. Vimentin is generally expressed in mesenchymal cells

and endothelial cells (ECs). In addition, vimentin is expressed and various tumor cells, and the up-regulation of vimentin is one among several markers for epithelial to mesenchymal transition (EMT) (Mendez, Kojima and Goldman, 2010).

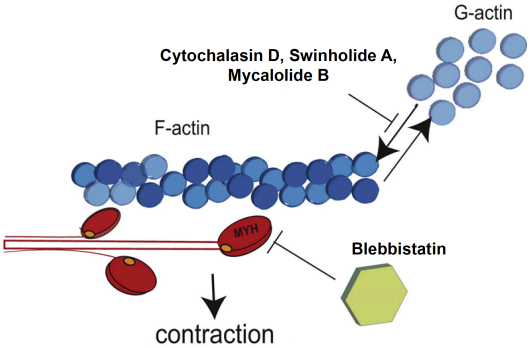
1.1.3.2 Microtubule (MT)

Microtubules are hollow cylinders of about 23 nm in diameter and consist of polymers of α/β-tubulin. They are essential to several cell processes, such as cell division, the transportation of cellular molecules, organelle distribution and cell mobility. Due to the relative rigidity of MTs, the cycles of polymerization and de-polymerization inherent to MTs could actually generate a push or pulling force through the dynamically extending or shrinking MT "tip" (Kent and Lele, 2017).

1.1.3.3 Actin filaments (AFs, also known as microfilaments)

Actin is the main component of the highly dynamic AF structures. Globular (G)-actin monomers combine as a linear polymer and continue to assemble into two intertwined chains called filamentous (F)-actin. AFs either connect with other AFs to form a bundle, or together with the motor proteins, such as myosin superfamily, to generate a network known as an actin cytoskeleton. The actin cytoskeleton facilitates the transmission and transduction of mechanical signals and at the same time, can generate intracellular tensions required for cell motility, muscle contraction, cell signaling and so on. This contractile property is supported by motor proteins, which exhibit distinct individual characters, as they are not only able to translocate along AF, but also induce the movement of AF itself.

Myosin II is the only member that can form polymeric assemblies with F-actin (Cheney, Riley and Mooseker, 1993). In skeletal muscle cells, myosin II forms strong thick-filaments to generate powerful contraction along with other proteins, however in non-muscle cells, myosin II associates with AFs to form "thinner" contractile actin bundles, called stress fibers (actomyosin fibers) (Kreis and Birchmeier, 1980). Stress fibers consist of connecting or connected to FAs, in which a cell attaches to the substrate in a highly regulated and dynamic manner, and they therefore contribute to a number of functions, such as cell migration and morphogenesis



As seen in ***Figure 4***, F-actin grows from the organization of G-actin monomers, which can be blocked by a polymerization inhibitor such as cytochalasin D (May *et al.*, 1998). The contraction force generated from myosin-actin can be abolished by the myosin II inhibitor blebbistatin (Straight *et al.*, 2003).

Figure 4. Schematic of inhibition of myosin-actin contractility. *Adapted and modified from (Chen et al., 2010). Reprinted with permission from the publisher.*

Unlike the well-documented dynamic organization of AFs in the cytoplasm, the regulation and function of the nuclear AF has recently been highlighted (Grosse and Vartiainen, 2013). A recent study revealed that physiological ligands for G protein-couple receptors (GFCRs) and Ca^{2+} elevation promoted nuclear F-actin assembly and altered chromatin dynamics (Wang *et al.*, 2019).

1.1.3.4 Rho signaling pathways

The Rho family of GTPases is one of the key mediators of the mechanical forces exerted on integrins and cadherins (Lessey, Guilluy and Burridge, 2012; Noren *et al.*, 2001). In this context, RhoA, Rac1, and Cdc42 have been intensively studied for their characteristic roles in actomyosin contraction (Arnold, Stephenson and Miller, 2017). RhoA cycles between GDP state (inactive) and GTP state (active). Rho-GTP are regulated via GEFs (guanine exchange factors) and GAPs (GTPase activating proteins) (Jaffe and Hall, 2005). The activation of RhoA positively affects mDia (mammalian homologue of Drosophila diaphanous, a formin protein) to directly nucleate and polymerize long AFs (Narumiya, Tanji and Ishizaki, 2009). Furthermore, Rho-GTP activates ROCK (Rho-associated coiled-coil forming kinase), which, in turn, phosphorylates the myosin light chain (Kimura *et al.*, 1996). RhoA thereby promotes the formation of the stress fibers crossing FAs, as well as cell-cell junctional tension (DeMali, Sun and Bui, 2014; Ratheesh *et al.*, 2012). Moreover, both Rac1 and Cdc42 are regulated by Rho GEFs and activate the arp2/3 complex through WAVE and N-WASP respectively, thereby promoting actin polymerization (Spiering and Hodgson, 2011). Cdc42 has been implicated as promoting the formation of the filopodia of migrating cells, while Rac1 has a greater role in lamellipodia formation.

Mechanical forces can trigger multiple signaling pathways, many of which result in regulating the activation of the GTPase RhoA, especially when the forces are relayed via cell adhesive molecules (Lessey, Guilluy and Burridge, 2012). A biphasic regulation of RhoA activity has been uncovered in ECs when subjected to flow *in vitro*: RhoA was inhibited 5–10 minutes after the shear stress was applied, but was gradually activated after 30 minutes (Tzima, 2006). Another example supporting the notion is that a more active form of RhoA was detected in cells subject to tensional stress produced by magnetic tweezers and applied perpendicularly (Zhao *et al.*, 2007). In addition to forces applied externally, the myosin-dependent contractility generated by cells adjusting to different ECM rigidity have an impact on RhoA activation (Wozniak *et al.*, 2003).

1.1.4 Force impact on nuclei

In 1997, the Ingber group discovered the molecular connection among integrins, F-actin and nuclear scaffolds as a systemic path for mechanical force transmission. From these and more recent data it is becoming increasingly clear that the nucleus is not only a storage site for hereditary information, but is also a rheostat that regulates mechanical strain (Maniotis, Chen and Ingber, 1997).

The mechanical force exerted on the integrins and cadherins on the cell surface is able to travel along the contractile cytoskeleton to be transmitted into distant a cytoplasm and nucleus, as shown in ***Figure 5*** (Wang, Tytell and Ingber, 2009). Compared to chemical signal propagation, a physical "stress wave" is 40 times more time efficient in provoking a nuclear response (Na *et al.*, 2008).

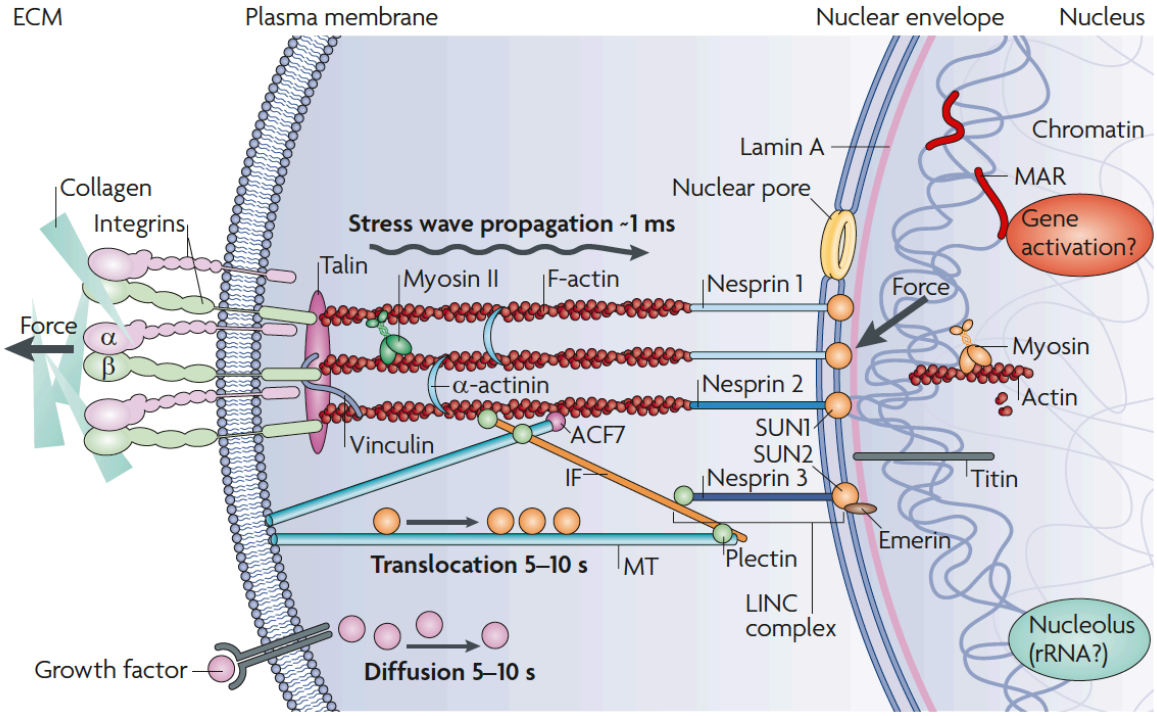


Figure 5. Molecular connectivity from the ECM to the nucleus. *A local force exerted on integrins from the ECM is concentrated at focal adhesions and conveyed to F-actin filaments. F-actins are connected to Nesprin 1 and 2 of the inner nuclear membrane protein SUN1/2. SUN-proteins directly bind to the lamins that form the lamina and nuclear scaffold, which affects chromatin and DNA organization and gene activation. The force channeled into the nuclear scaffold might take milliseconds. By contrast, it takes seconds for the chemical diffusion of growth factors to alter nuclear shape and function. Adapted from (Wang, Tytell and Ingber, 2009). Reprinted with permission from the publisher.*

1.1.4.1 Nuclear shape and positioning

When a certain force is exerted through the cytoskeleton onto the nucleus, whether from contractile energy for migration or tensions from adjacent cells, nuclear properties generally respond in two manners: to deform or to move.

Depending on the cell type, the cell cycle stage, the migratory stage and differentiation status, nuclear shape can differ dramatically (Gundersen and Worman, 2013a); however, in most cases, nuclear shape coincides with the shape of the cell. Direct evidence came from an observation that ECs on an elongated pattern forces the elongation of the nuclei (Versaevel, Grevesse and Gabriele, 2012). This suggests a mechanical model, in which stress fibers induced by force orient the cell along the force direction and therefore elongate the cell nucleus. The rounding of the cell shape that is induced by drugs that inhibit myosin II activity

or actin filament formation can also result in the rounding of the nuclei (Buxboim *et al.*, 2017).

The position of nuclei in cells is regulated by cell geometry and activity. When the nucleus remains in a stationary position, the forces acting on it reach a balance. A number of studies have shown *in vitro* that cell positioning can be altered by a disrupted force balance on the nucleus, manipulation of cytoskeletal contractility and the mutation of the nuclear anchoring proteins, as reviewed in (Lele, Dickinson and Gundersen, 2018; Gundersen and Worman, 2013b).

1.1.4.2 The cellular nucleus responds to mechanical forces

Similarly to the outer cell membrane, which forms a physical boundary permitting the transfer of bio-chemical and -mechanical signals, the nuclear lamina separates the cytoplasm from the nucleoplasm (Gruenbaum and Medalia, 2015; Butin-Israeli *et al.*, 2012). The nuclear membrane incudes proteins that connect to the cytoskeleton as well as proteins that regulate mRNA and protein transport. The proteins involved in nuclear positioning and mechanotransduction are described below.

The linker of nucleoskeleton and cytoskeleton (LINC) complex, similar to FAs and AJs at the outer membrane, functions as a bridge through which mechanical signals can pass into the nucleus. This physical attachment to the cytoskeleton ensures proper nuclear shape and subcellular nuclear positioning (Lee and Burke, 2018; Hieda, 2019; Gundersen and Worman, 2013a). The LINC complexes are comprised of nuclear envelope spectrin repeat proteins (Nesprins) located on the outer nuclear surface and SUN proteins (Sad1/UNC-84) at the inner the nuclear membrane. The cytoskeleton in cytoplasm is therefore able to physically couple with lamins and chromatin inside nucleus through SUN/Lamin A and Nesprin/Actin associations (Starr and Han, 2002; Haque *et al.*, 2006; Starr and Fischer, 2005).

Lamins, such as Lamin A/C (two isoforms coded by gene *LMNA*) and Lamin B cover the interior of the nuclear envelope. They are a type V intermediate filament and thus, structurally support the nucleus in resisting mechanical perturbations. Dramatic changes in the nucleus is the hallmark of *LMNA* genetic mutations that leads to laminopathies, such as Hutchinon-Gilford progeria syndrome (Gonzalo, Kreienkamp and Askjaer, 2017). Moreover, nuclear stiffness is determined by the relative levels of Lamin A/C. It was recently shown that the stiffness of the individual organ also determines nuclear stiffness by regulating *LMNA* levels (Swift *et al.*, 2013).

Interestingly, chromatin have been proposed to function as a physical spring to push or pull against nuclear deformation, depending on a restoring force generated by linker DNA and nucleosome interaction via histone tails (Maeshima, Tamura and Shimamoto, 2018).

1.1.5 YAP is a mechanoresponsive transcription factor

Yes-associated protein (YAP, also known as YAP1 or YAP65) and the transcriptional co-activator with PDZ-binding motif (TAZ) are downstream effectors of the Hippo pathway. YAP activity is tightly regulated by the MST1/2 kinases, which can trigger phosphorylation and activation of LATS1/2 kinases, which further phosphorylate and inactivate YAP and TAZ, thus preventing nuclear relocation (Gaspar and Tapon, 2014). Most of YAP/TAZ transcription targets are associated with cell proliferation, and the activity of Hippo molecules in the nucleus has therefore been associated with organ growth and tumor progression (Zanconato *et al.*, 2015). Remarkably, recent studies have revealed a rheostat of mechanical force; that is, YAP is able to directly promote the transcription of genes related to ECM composition, cell–ECM communication and cytoskeletal integrity (Nardone *et al.*, 2017; Morikawa *et al.*, 2015).

In response to the mechanical cues from ECM or cells in their vicinity, YAP/TAZ can translocate into the nucleus to activate the specific transcription factors and thereby affect gene expression. More specifically, when the cells are mechanically activated by a large adhesive area, stiff substrate, low cell density, disturbed flow and stretching of the confluent monolayer, YAP/TAZ enter the nucleus to regulate gene expression (Panciera *et al.*, 2017). Thus, YAP/TAZ not only sense the mechanics but also mediate the biological effect in different biological contexts by modification on transcriptome, for example neuronal differentiation, apoptosis et cetera.

Moreover, YAP/TAZ-based mechanotransduction demands the integrity of the actin cytoskeleton. At least in *in vitro* experiments, YAP/TAZ activity is decreased by F-actin inhibition (Dupont *et al.*, 2011; Morikawa *et al.*, 2015; Piccolo, Dupont and Cordenonsi, 2014).

There is increasing evidence for the clinical importance of the proper control of mechanotransduction: Genetic mutations of the key molecules and aberrant activation of relevant signaling pathways may lead to severe clinical problems, such as fibrosis, myopathies and cancer (Jaalouk and Lammerding, 2009). Strikingly, many of these defects may be directly or indirectly caused by the deregulation of YAP/TAZ (Panciera *et al.*, 2017).

Endothelial cells are intimately exposed to mechanical forces generated by blood flow and alteration in mechanical parameters may directly initiate the vascular pathogenesis (Hahn and Schwartz, 2009). Normally, YAP/TAZ are inhibited in ECs that are cultured under laminar flow. However, disturbed shear stress activates the YAP/TAZ and JNK (c-Jun N-terminal kinase) signaling pathway, and thus up-regulates a number of pro-inflammatory genes whose expression results in atherosclerosis (Wang *et al.*, 2016b).

Tissue fibrosis may occur in the liver, lungs, kidneys and other organs. This is characterized by excessive stiffness in ECMs by over-activated fibroblasts (or myofibroblasts) over a certain time. In liver fibrosis, the activation of YAP in hepatic cells results in liver damage (Mannaerts *et al.*, 2015). In idiopathic pulmonary fibrosis, YAP/TAZ activity maintains a

pro-fibrotic regulation of transcriptome on lung fibroblasts, which increases the Fn and collagen deposition (Jorgenson *et al.*, 2017).

Finally YAP/TAZ pervasive activation has been uncovered in various human cancers, which results in massive proliferation, drug resistance and metastasis (Piccolo, Dupont and Cordenonsi, 2014; Zanconato, Cordenonsi and Piccolo, 2016).

1.1.6 Force measurement and manipulation

Whether the mechanical forces are generated from cell–ECM or cell–cell junctions, the subcellular forces that are relayed are as minute as in picoNewton (pN) range. For instance, 80–300 pN is necessary to unfold Fn type III domain (Oberhauser *et al.*, 2002), and the forces generated by growing microtubules, F-actin-binding myosin motors and microtubule-binding motor proteins only require 3–7 pN (Cost *et al.*, 2015). Despite apparent difficulties in measuring the forces *in vivo*, a number of approaches have been developed to scrutinize cellular mechanical tension in living cells *in vitro* (Ungai-Salánki *et al.*, 2019; Roca-Cusachs, Conte and Trepat, 2017). Due to space limitation, this section only focuses on several well-established and representative approaches.

1.1.6.1 Force sensors based on known material

"Cantilevers"- based methods

These systems apply cantilevers with a known stiffness to the bottom layers and allow cells or tissue to grow on top. Simply speaking, the more the cells contract, and the harder those cantilevers bend. By measuring the observed displacements of the free end of pillars, we can calculate the contractile energy and force (Polacheck and Chen, 2016). The micro-pillars can be made of a variety of materials, such as magnetic beads, molecules and collagen, among which traction force microscopy (TFM) employs fluorescent beads emerged in Fn-coated elastic substrate for cells to grow on. To date, TFM has improved its accuracy and spatial resolution dramatically and become one of the most extensively used methods of measuring cell–ECM contractile force (Plotnikov *et al.*, 2014).

Surface Plasmon Resonance Microscopy

Surface plasmon resonance (SPR) microscopy is an approach for cell–substrate contacts of cells in culture based on the optical excitation of surface plasmons (Giebel *et al.*, 1999). Without any pre-engineering, cells are cultured on the coated glass prism with a laser beam in below. The evanescent field generated by the electromagnetic wave can be interfered by the cells, so that the intensity of reflected light changes corresponding to the distance between cell and substrate, and this can be correlated to the local force.

Förster resonance energy transfer-based tension sensors

Förster resonance energy transfer-based tension (FRET) sensors offer an excellent tool for mapping the force distribution inside living cells with high resolution (Grashoff *et al.*, 2010). In brief, the FRET sensor is recombined into a specific protein in a transgenic cell type, and

another gene is constructed to express two fluorophores with an elastic linker. Once the force exerted, the linker would extend like a spring under force, and in this way the change in distance between fluorescent proteins can be captured by fluorescent microscopy.

1.1.6.2 To apply force exogenously

From another perspective, researches, both biologists and physicists, have been developing systems to actively manipulate the force applied to the cells and observe or measure the cell response.

Flow chambers

Aiming to recapitulate the *in vivo* physiological/pathological flow condition *in vitro*, we often apply external shear sheer on ECs in vascular biology research. The Ibidi flow system includes the advantages of various flow conditions, ease of operation, minimal external supply needed and possibilities for more applications.

Forces applied by physical stretching or touching

The stretchable/elastic culture substrate provides a useful tool to access the cell resistance against external strain. The cells are cultured on a stretchable substrate, which enables artificial straining and relaxing of the elastic membrane.

Atomic force microscopy (AFM), micropipette aspiration, microplate, magnetic and optical tweezers apply force directly through specifically coated beads or a "tip" on the cell membrane or subcellular structures. They are useful for either probing the cellular reaction to physical stimuli or measuring exact mechanical quantity (Sugimura, Lenne and Graner, 2016).

1.1.6.3 Cell itself as a sensor

Instead of "human-made" force sensors, a "cell-made" device, to some extent, provides more "natural" and dynamic data. Those sensors require a known property and exhibit noticeable conformational changes on force application (Roca-Cusachs, Conte and Trepat, 2017). Molecular conformational changes include an unfold-fold switch, domain re-orientation, the opening of ion channels et cetera (Ehrlicher *et al.*, 2011; Yao *et al.*, 2016; Wu, Goyal and Grandl, 2016). With the help of approaches that precisely manipulate the force, many protein conformational changes have been identified. For example, the application of external forces induced the stretching of single talin rods, which unmasked cryptic binding sites that permitted vinculin binding (Rio *et al.*, 2009). Similarly, it has been reported that force could induce a conformational switch of α-catenin, which activated vinculin binding (Yao *et al.*, 2014b). Moreover, based on the known conformation changes of force-sensitive molecules, external probes can be designed to distinguish those changes as a "force detector." Krieger and colleagues labeled cysteine residues and found that this cysteine labeling in several proteins, changed by mechanical input in living cells (Krieger *et al.*, 2011).

1.2 Mechanical force in vascular development

During vascular development, the endothelium, which forms the inner layer of blood vessels, relentlessly encounters numerous and varied mechanical forces derived from ECM, interactions with adjacent cells, pulsatile flowing blood and extravasation of cells from flowing blood (***Figure 6***). How ECs sense and respond to those mechanical inputs is essential for new blood vessel formation, vessel maturation and eventually a functioning blood vessel network.

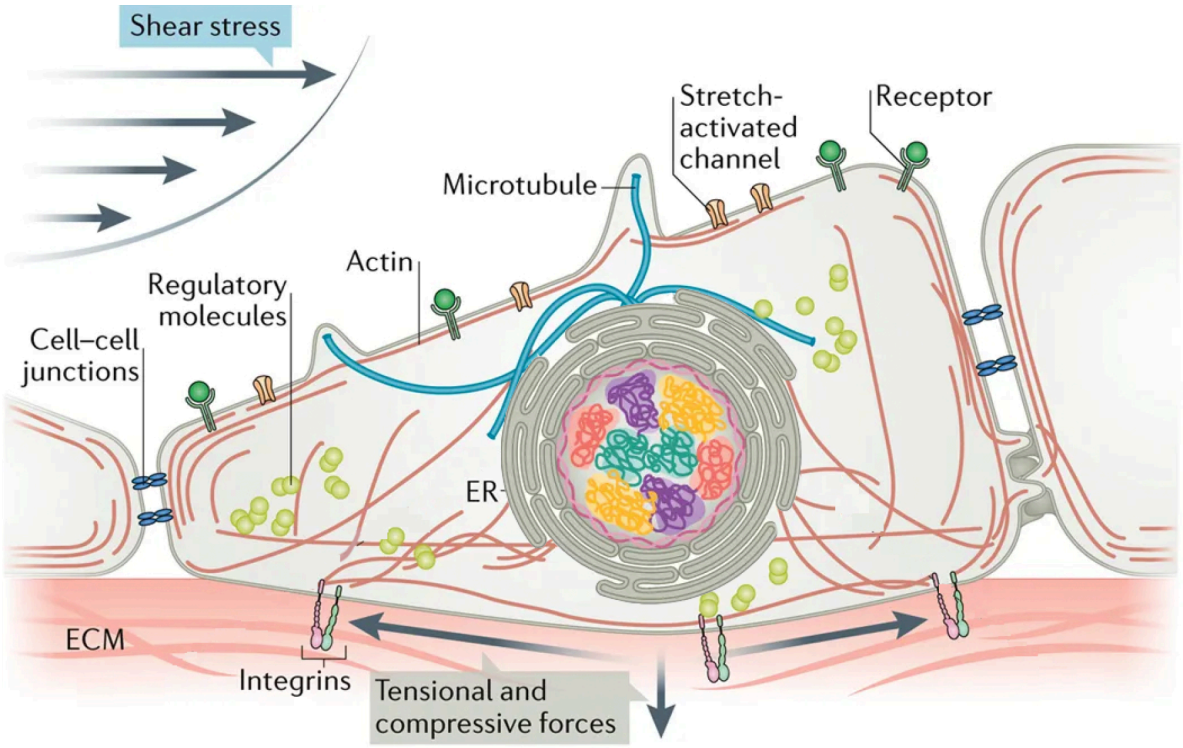


Figure 6. ECs subjected to physical forces. *ECs feel the force from the ECM, cell-cell junction and direct encounter of the shear stress from pulsatile flow. Adapted and modified from (Uhler and Shivashankar, 2017). (Uhler and Shivashankar, 2017). Reprinted with permission from the publisher.*

1.2.1 New blood vessel formation

New blood vessels are formed by three different mechanisms: 1) Vasculogenesis: This is involved in the establishment of the first primary vessels during embryogenesis. In this process, the aorta and the cardinal vein are formed by the differentiation of EC from mesoderm progenitor cells. 2) Intussusception: The capillary wall extends into the lumen and thus splits a single vessel into two. 3) Angiogenesis: This process describes the formation of blood vessels from the pre-existing ones. More specifically, vascular growth involves tip cell selection, migration, stalk cell proliferation, and ultimately vascular stabilization. Uncontrolled angiogenesis precedes a wide spectrum of vascular pathologies such as kidney disease, cardiovascular complications, autoimmune syndromes et cetera. Angiogenesis is also a hallmark of cancer progression as the growing tumor is in increasing demand of nutrients and oxygen to expand beyond a limited size of 500 μm in diameter. The hypothesis that tumor angiogenesis can be targeted as a cancer therapy was proposed by Judah Folkman in

1971 (Folkman, 1971). Since then, anti-angiogenic therapies have entered the clinic, though with only modest clinical efficacy.

1.2.1.1 Force transmission during angiogenesis

During angiogenesis, ECs migrate in a directional manner guided by growth factors. Migration involves the separation of EC into tip cells and stalk cells. As its name suggests, the endothelial tip cell is a specialized EC that leads to and guides the endothelial sprout. They are highly polarized with numerous extended protrusions (termed filopodia) to sense and probe the environment. These filopodia not only detect and respond to chemotactic factors such as vascular endothelial growth factor (VEGF)-A and axon guidance factors but also mechanically interact with the surrounding ECM. The ECM exerts mechanical control on the endothelium via integrin-based FAs that relay forces to the cellular cytoskeleton (Hynes, 2002; Schwartz, 2010b). These FAs bridge the cytoskeleton and ECM resulting in stress fibers of myosin-actin filaments pulling the cell forward (Lamalice, Le Boeuf and Huot, 2007).

Fn is a major component of the ECM. Among many alternatively spliced variants of Fn, the exons EIIIB (between Fn repeats III_7 and III_8) and EIIIA (III_{11} and III_{12}) are strongly associated with vascular remodeling (Pankov and Yamada, 2002). Fn containing these two domains is highly expressed in newly formed vessels during angiogenesis, but it is barely detectable in mature vessels in adults (Murphy, Begum and Hynes, 2015). The Fn-binding endothelial integrins α5β1- and αvβ3/β5-integrin receptors are up regulated in newly forming vessels and are therefore considered anti-angiogenic targets. However, the mechanisms whereby these molecular pathways control angiogenesis are complex (Alday-Parejo, Stupp and Rüegg, 2019). Inactivation of integrin α5 and αv during mouse development causes death *in utero* due to vascular defects (Murphy, Begum and Hynes, 2015). Although highly expressed in tumor endothelium, conditional deletion of Fn, α5β1 and αvβ3/β5 does not have a satisfactory outcome in inhibiting tumor expansion (Murphy, Begum and Hynes, 2015), which suggests the up-regulation of alternative compensatory pathways. Indeed, analysis of the integrin adhesome has identified over 180 proteins, some of which are regulated by actin tension (Zaidel-Bar and Geiger, 2010; Schiller *et al.*, 2011). Further insight into how mechanical forces are transduced may develop a better understanding of the mechanisms of endothelial tip cell migration during angiogenesis.

1.2.1.2 EC-perivascular cell interaction

Endothelial cells are the primary building block of blood vessel construction, however it is insufficient for ECs to from functional vasculature independently. When the vessel sprouts fuse with neighboring ones to form capillary loops, they require help from other supporting perivascular cells, such as smooth muscle cells and pericyte.

Vascular smooth muscles cells commonly support larger arteries and veins by wrapping the vessels in a circumferential manner. In contrast, pericyte characterized by an elongated and flattened shape, specifically embrace ECs at smaller capillaries. By multiple projections

covering the surface of ECs, pericyte form direct contact to EC in addition to paracrine signaling communications. Adhesion plagues anchoring pericyte to ECs are centered by the N-cadherin associated complex, which enables transmission of mechanical contractile force in between (Gerhardt, Wolburg and Redies, 2000).

Platelet derived growth factor (PDGF) is released primarily by ECs and activates the PDGF receptor β (PDGFR-β) on pericytes, among other perivascular cells. Genetic ablation of PDGF or PDGFR-β in mice causes embryonic death due to dysfunctional vasculature with an enlarged, highly permeable and dilated morphology (Lindahl *et al.*, 1997). Poor coverage of pericyte on ECs was observed histologically, as was EC hyperplasia, which supports the notion that pericyte plays a key role in stabilizing developing vessels and preventing ECs from redundant angiogenesis (Gerhardt and Betsholtz, 2003).

1.2.2 The cardiovascular system

In the human body, the cardiovascular system consists of the heart, blood vessels and approximately five liters of blood. Most, if not all, organs are dependent on this organized circulatory system, which is responsible for the transport of oxygen and nutrients. Regardless of vessel type, endothelium undergoes multiple mechanical forces that are exerted on the vessel wall (radial, circumferential and longitudinal forces) or on the endothelial surface (shear stress). However the magnitude of those forces varies across a wide range on arteries and veins; for example, the aorta sustains 10-fold more shear stress (dyn/cm^2) on average than the vena cava (Givens and Tzima, 2016). In return, ECs in arteries and veins exhibit distinct morphological and functional differences in response.

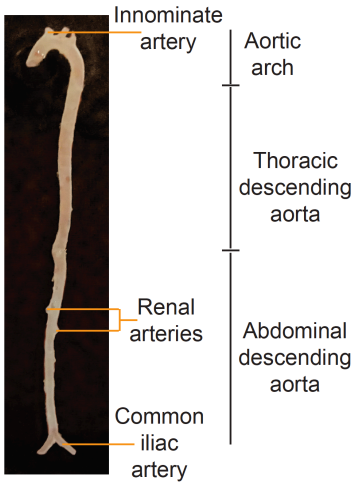
1.2.2.1 Artery and vein

Arteries, in general, are smaller in diameter than veins but mostly show much thicker vessel walls. The innermost layer of the vessel is the *tunica intima* (intima for short), which contains endothelium and a supportive matrix composed of collagen and elastin. The elastic fibers form a specialized internal elastic lamina in arteries, especially abundant in larger ones such as the aorta. This lamina provides reversible elasticity that allows large arteries to expand and relax with the cardiac cycles (Cocciolone *et al.*, 2018).

Aside from wall structure, distinct morphological differences exist between arterial and venous ECs: Arterial ECs are elongated and aligned in the direction of laminar flow, while venous ECs exhibit a more rounded phenotype. Furthermore, veins are more vulnerable to inflammation and leukocyte trafficking, especially in the post-capillary venules (dela Paz and D'Amore, 2009).

1.2.2.2 Hemodynamics of the aorta

The aorta is the main artery in the body and thus exposed to the highest pulsatile blood pressure.



In an anatomical view, the aorta consists of the transverse aortic arch (AA) and straight thoracic and the abdominal descending aorta (DA), as shown in ***Figure 7***. The major branches of the whole aorta include the innominate artery (first arterial branch in AA), renal arteries in the abdominal DA and the common iliac artery from the bifurcation of the DA.

Figure 7. Anatomical view of a mouse aorta. *Full-length mouse aorta was dissected under stereomicroscope and photographed by cell phone. The anatomical terms are labeled in the image.*

During the systole of the cardiac cycle, the blood is pumped out at the peak velocity. As the path starts to curve at an ascending aorta of AA, the blood going along the outer curvature tends to change direction towards the inner wall, resulting in a helical rotating of the flow. After passing the arch, the spiral flow turns into a laminar shear straight down to the DA. This dynamic flow pattern is repeated with each impulse of the heart. Understanding the hemodynamics of the aorta provides the fundamental basis for exploring EC biology in different parts of aorta.

1.2.2.3 Mechanosensors at EC membrane

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is an adhesion molecule located at the cell membrane that mediates homophilic contact with adjacent ECs. Early evidence has shown that fluid shear stress induces PECAM-1 phosphorylation, Shp2/ Gab1 recruitment and the activation of ERK (Osawa *et al.*, 2002). Together with VE-cadherin and VEGFR2, PECAM-1 has been identified as a mechanosensing complex that mediates several shear stress responses, including EC alignment, NF-kB activation et cetera (Tzima *et al.*, 2005).

Integrins are capable of sensing minute changes in shear stress or cytoskeletal contractility, even though shear stress has no direct impact on the basal membrane (Guilluy, Dubash and Garcia-Mata, 2011). Integrin activation when subjected to shear induces integrin clustering, which is required for RhoGTPases and NF-kB activation. Integrins are an integral part of the response to mechanical force in ECs (Givens and Tzima, 2016).

1.2.2.4 Arterial diseases

Cardiovascular disease (CAD) remains the number one cause of mortality worldwide accounting for over 30% of all global deaths *(data from https://ourworldindata.org/causes-of-death)*. Arterial diseases, such as atherosclerosis and abdominal aortic aneurysm (AAA), are the most common but most life-threatening types, and they can be fatal due to the acute ischemia of vital organs or massive hemorrhage into the peritoneal cavity.

Atherosclerosis

Atherosclerosis describes a chronic process leading to the narrowing and hardening of arteries due to the build-up of plaque. When the lumen is clogged, the consequence can be lethal depending on which arteries are affected. For instance, myocardial infarction is a serious consequence of coronary atherosclerosis.

Aging and hypercholesterolemia are well known risk factors in atherosclerosis. Furthermore, atherosclerosis is linked to shear stress, as plaque formation generally occurs at sites with disturbed flow such as blood vessel bifurcations (Kwak *et al.*, 2014; Cheng *et al.*, 2006; Dai *et al.*, 2004). Studies using unbiased systematic approaches (transcriptomics and proteomics) have revealed multiple pathways commonly regulated by altered shear stress conditions (Souilhol *et al.*, 2019); these include bone morphogenetic protein (BMP) signaling (belonging to the TGFβ superfamily), hypoxia-inducible factor (HIF)-α, Notch, WNT, the homebox (HOX) family, the Hippo-YAP/TAZ pathway et cetera. For example, YAP is localized in the nucleus to a greater degree in areas exposed to disturbed rather than undisturbed flow in the mouse aorta (Wang *et al.*, 2016a). In return, laminar flow inhibits YAP and TAZ activation (Wang *et al.*, 2016c).

Biochemical and biomechanical cues or damage to the endothelial layer may trigger the accumulation of circulating cytokines and, as a consequence, leukocyte extravasation as shown in ***Figure 8***. Once monocytes transmigrate the endothelium, they begin to differentiate into macrophages, taking up oxidized low-density lipoproteins (oxLDL) and transforming into foam cells, which are the key component of atherosclerotic plague (Tajbakhsh *et al.*, 2018; Chistiakov *et al.*, 2017).

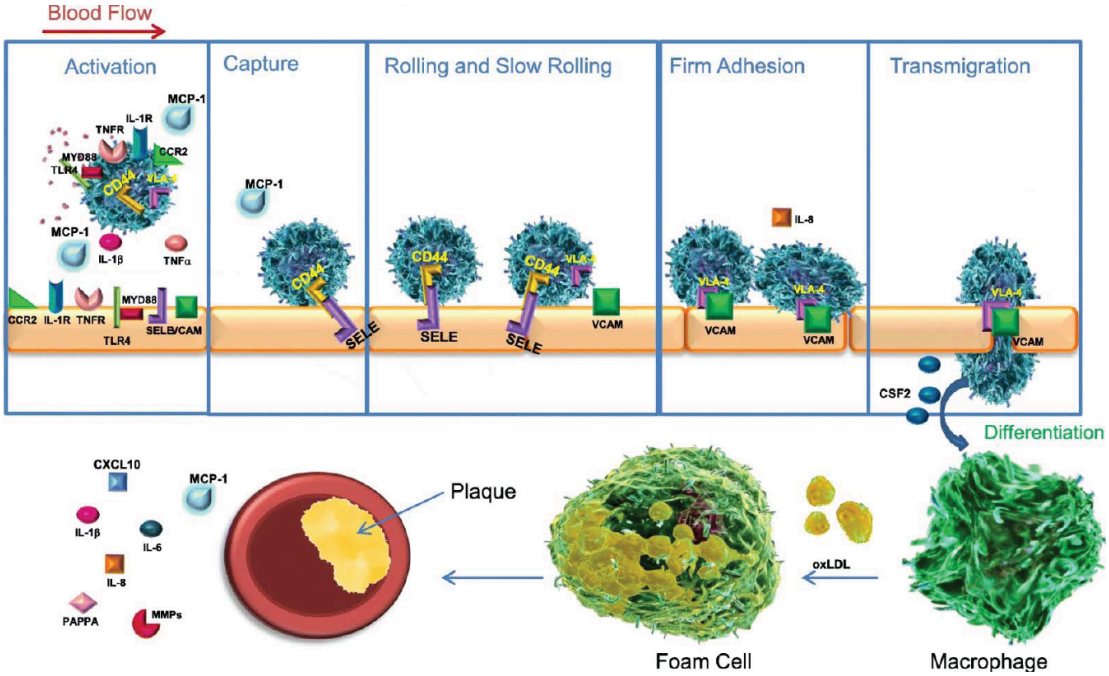


Figure 8. Progressive process of atherogenesis. *The steps involved in the formation of atherosclerosis include: endothelium activation, leukocyte capture, rolling and adhesion, macrophage differentiation, and finally plaque development. Several selected cytokines and molecules are labeled*

in the image, but it is not complete. Adapted from open-access article (Tsujikawa et al., 2019). Reprinted with permission from the publisher.

Abdominal aortic aneurysm

An AAA in humans is not a rare disease, presenting in up to 8% of the male population over the age of 65 and aneurysm rupture accounts for approximately 7,000 male deaths per year in the United States alone (Nordon *et al.*, 2010). A sexual dimorphism has been reported by epidemiological reports in the development of AAA; that is, the prevalence of AAA in the population of males over 50 is 4–5 times greater than in women of the same age (Hannawa, Eliason and Upchurch, 2009; Katz, Stanley and Zelenock, 1997); however, women are no longer protected after post-menopause (Villard *et al.*, 2011), suggesting that an endogenous sexual hormone underlines the sex differences in AAA (Boese *et al.*, 2018).

Ruptured AAA has an extremely high mortality rate (80%), and surgical repair with stents is currently the only available treatment (Golledge *et al.*, 2019). The slow development of medical therapy for AAA is partially attributed to the lack of insights into its molecular mechanism, despite various mouse models for AAA studies (Lareyre *et al.*, 2017; Angelov, Zhu and Dichek, 2017).

Generally, there are three stages of AAA development: initiation, progression, and rupture, with different mechanisms implicated in human aortic aneurysm pathogenesis (Thompson *et al.*, 2008). ***Table 2*** provides a brief summary, showing that atherosclerosis and immune response are closely associated with AAA initiation and that vascular inflammation is therefore a high risk factor for aneurysm occurrence (Forester *et al.*, 2005; Golledge *et al.*, 2006). Since 2006, the American Heart Association guidelines have suggested AAA as an atherosclerotic equivalent (Hirsch *et al.*, 2006). What is also apparent is that biomechanical forces are potential drivers of every stage of aneurysm development. Excessive force caused, for example by hypertension, is a risk factor of AAA in both human and rodent studies (Golledge *et al.*, 2006; Kanematsu *et al.*, 2010). However, locally increased shear stress and wall tension can inhibit early aortic enlargement due to the reductions in oxidative stress (Nakahashi *et al.*, 2002). Overall the forces need to be fine-tuned to maintain vascular homeostasis.

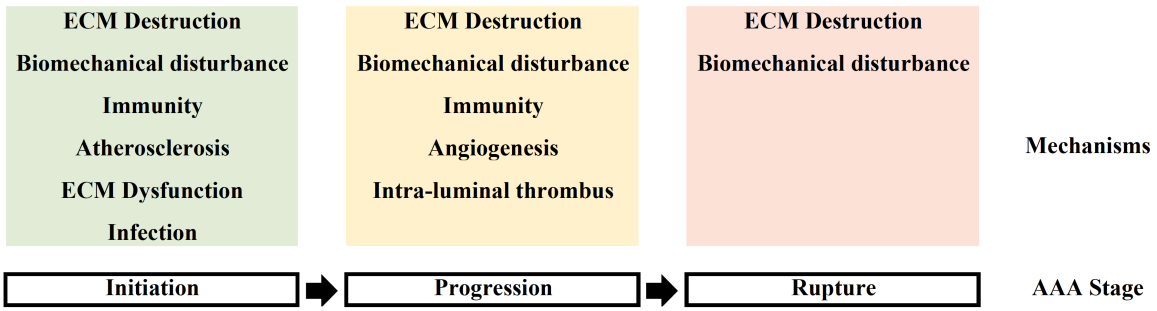


Table 2. Mechanisms implicated in AAA initiation, progression and rupture. *Summarized from (Golledge, Shi and Norman, 2011). Illustrated by Olof Andersson.*

1.3 Angiomotin: A scaffold protein family

The Angiomotins belong to a family of scaffold proteins that consists of three members Angiomotin (Amot), Angiomotin-like 1 (AmotL1) and Angiomotin-like 2 (AmotL2). These three members are characterized by a WW-binding motif Coiled-coil (C-C) domain and a PDZ-binding motif. Amot, but not AmotL1 or AmotL2, contains an angiostatin-binding domain between C-C domains and the PDZ binding motif. Each Amot consists of one full-length isoform and a shorter variant, including p130/p80 Amot, p100/p90 AmotL1 and p100/p60 AmotL2 (Bratt *et al.*, 2002; Nishimura *et al.*, 2002).

1.3.1 A brief history of the discovery of Amot proteins

Amot (p80) was identified in 2001 as a membrane associated protein-binding angiostatin, which is the endogenous angiogenesis inhibitor circulating in the blood (Troyanovsky *et al.*, 2001). In 2006, the longer isoform of p130 Amot was reported in different cell types, including vascular ECs, whose N-terminal extension mediated the association with actin filaments. The transfection of p130 Amot induced the F-actin formation and the cell's elongation in shape (Ernkvist *et al.*, 2006). AmotL1 (p100) was identified from TJ protein screening using a fluorescence localization-based expression cloning method and is thus, also known as TJ-enriched and associated protein (Nishimura *et al.*, 2002). Another short isoform of p90 AmotL1 was articulated to regulate EC polarity and junctional stability during blood vessel formation (Zheng *et al.*, 2009). So far, no functional differences between p100 and p90 isoforms have been reported. The last member, AmotL2 (p100, also known as LCCP or MASCOT), was discovered via homology searches and the assembly of data from GenBank and was included in the Amot family. Over several decades, numerous studies have uncovered the binding partners with those characterized domains, as summarized in the ***Figure 9***.

1.3.2 Amot proteins in vascular development

Given that Amots were originally discovered as regulators of EC migration, a number of studies have focused on the role of Amot proteins in vascular development. Amot was initially discovered to mediate the angiostatin inhibition of EC migration and tube formation *in vitro* (Bratt *et al.*, 2002; Levchenko *et al.*, 2003). During mouse embryogenesis, most Amot knockout embryos (of 129/SvEv mouse strain) died soon after gastrulation with abnormal furrows of visceral endoderm as a result of the restraint of cell migration (Shimono and Behringer, 2003). In mice mixed between 129/SvEv and C57/B6, 75% of *amot-null* mice died between E11 and E15 due to severely insufficient vasculature in the intersomitic vessels and highly dilated brain vasculature (Aase *et al.*, 2007). Furthermore, Amot knockdown endothelial tip cells in zebrafish became less migratory and exhibited a reduction in the number of filopodia, suggesting an anti-angiogenic effect (Aase *et al.*, 2007). Antibodies targeting Amot using DNA vaccination and a human monoclonal antibody B06 have been generated as a result, and both have successfully inhibited tumor growth by blocking angiogenesis (Holmgren *et al.*, 2006; Levchenko *et al.*, 2008).

Since AmotL1 is 62–64% homologous to Amot, they were assumed to have certain overlapping functions. For example, an *in vitro* study showed that both AmotL1 and Amot promoted EC migration and influenced cell junction stability (Aase *et al.*, 2007; Zheng *et al.*, 2009). However, they also displayed distinct functions in *in vivo* studies. Using a zebrafish model, our group verified that Amot controls EC migration during new blood vessel sprouting, whereas AmotL1 functions primarily to stabilize the cell–cell junction in stalk cells (Zheng *et al.*, 2009).

Similarly, the knockdown of AmotL2 in zebrafish resulted in impaired cell movement in mosaic embryos as a result of failed translocation of phosphorylated c-Src (Huang *et al.*, 2007). Later, AmotL2 has been demonstrated to link radial actin filaments to VE-cadherin junctional complex. These complexes transduce force through the cellular junctions and inactivation of *amotl2* in mouse and zebrafish embryos abrogates aortic lumen expansion resulting in death *in utero* at embryonic day 10 (Hultin *et al.*, 2014). A similar function of AmotL2 was uncovered in epithelial cells, in which AmotL2 connects to E-cadherin, organizes radial actin filaments, and is crucial for blastocyst hatching in pre-implantation mouse embryos (Hildebrand *et al.*, 2017). In epithelium, hypoxia-induced p60 AmotL2 was identified in invasive tumor cells, promoting cell migration and metastasis (Mojallal *et al.*, 2014). However this isoform was not found expressed in vascular endothelium.

1.3.3 Binding partners of Amot proteins

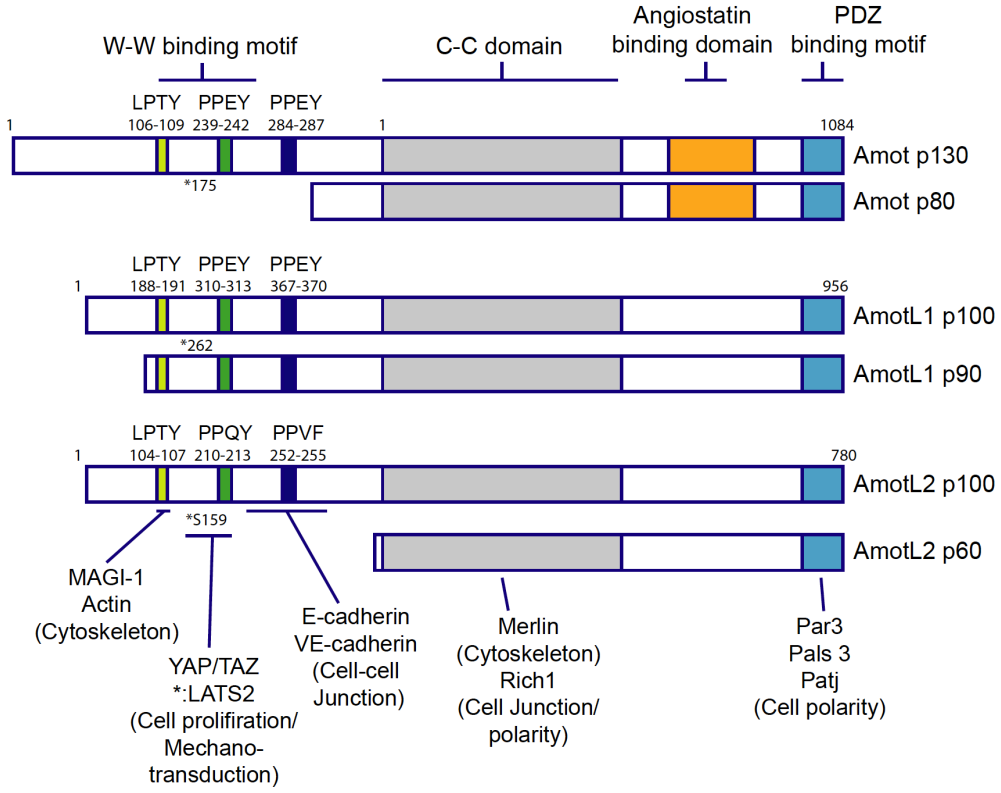


Figure 9. Schematic of Angiomotin protein family. *This scaffold protein family is composed of Amot, AmotL1 and AmotL2, each of which consists of two isoforms. The N-terminal conserved glutamine-rich domains: LPTY binds cytoskeletal proteins MAG1-1 1 (Bratt et al., 2005; Hildebrand et al.,*

*2017) and actin (Ernkvist et al., 2006); PPEY and PPQY are the sites for YAP and MST2 bindings (Mana-Capelli et al., 2014; Zhao et al., 2011); * depicts LATS1/2-dependent phosphorylation sites for of all Amots (Serine 176 for Amot for example) (Paramasivam et al., 2011; Chan et al., 2013); finally, the 220aa-307aa in AmotL2 p100 isoform has been identified to bind E-cadherin in epithelial cells and VE-cadherin in ECs. The C-C domain is associated with Merlin (Yi et al., 2011) and Rich1 (Wells et al., 2006). Amot (p130/p80) contains an angiostatin-binding domain (Bratt et al., 2005). The PDZ binding motif at the C-terminal binds to the apical polarity proteins Par3, Pals1 and Patj (Ernkvist et al., 2009; Wells et al., 2006).*

1.3.3.1 Hippo signaling pathway

Classically, the Hippo pathway regulates cell proliferation, apoptosis and tissue formation, and it is intimately regulated by cell geometry. The signaling cascade includes MST and LATS-kinases, positively regulating the downstream transcriptional co-activators YAP and TAZ. Phosphorylated YAP is prevented from entering the nucleus, where it binds to the DNA-binding TEA domain (TEAD), to be transcriptionally active and drives the transcriptional programs that promote proliferation and inhibit apoptosis. Amot was first described as a YAP binding partner from a global proteomic data analyzing human deubiquitinating enzyme interaction (Sowa *et al.*, 2009). Later, LPTY and the first PPEY motif from Amot and AmotL1, as well as the PPXY motif in AmotL2, were discovered to directly bind YAP (Moleirinho, Guerrant and Kissil, 2014).

The direct binding of Amots/YAP has a high impact on YAP/TAZ subcellular localization. Overexpression of Amots in epithelial cell lines (HeLa and MCF7) induced YAP/TAZ retention in the cytoplasm (Wang, Huang and Chen, 2011; Zhao *et al.*, 2011). However, the knockdown of Amot or AmotL2 in MDCK and MCF10A cells significantly increased the nuclear localization of YAP/TAZ, as well as the activation of YAP target genes evidenced by the up-regulation of *CTGF* and *CYR61 (Zhao et al., 2011; Chan et al., 2013)*. In contrast to these findings supporting the negative regulation of YAP by the Amot family, p130 is required for YAP function, as described by Yi and colleagues in *in vivo* experiments, where liver-specific Amot knockout promoted tumorgenesis. The possible mechanisms might be that Amot inhibited YAP phosphorylation by competing binding domains to LATS1 in cytoplasm, as well as facilitating YAP within the nucleus to regulate downstream genes (Yi *et al.*, 2013).

The Hippo pathway can regulate the post-translational modification of Amot proteins, for example phosphorylation. Multiple experiments have confirmed the LATS1/2-dependent phosphorylation of Amot (serine 175), AmotL1 (s262), and AmotL2 (s159) at N-terminal WW binding domains (Hirate *et al.*, 2013; Adler *et al.*, 2013; Dai *et al.*, 2013; Chan *et al.*, 2013). In the context of neurobiology, LATS1 has been recently indicated to be a neuronal regulator of p130 Amot by specifically phosphorylating the N-terminal sites of s175 during brain development (Wigerius *et al.*, 2018).

1.3.3.2 The associations with cell polarity, junctional and cytoskeletal proteins

Several pieces of evidence have reported the importance of the C-terminal PDZ binding motif for Amots function in promoting EC migration. Knockdown only PDZ motifs in Amot and

AmotL2 was sufficient to limit EC migration in zebrafish embryos and the endothelial-specific deletion of Amot PDZ binding domains led to embryonic death by E9.5 (Huang *et al.*, 2007; Ernkvist *et al.*, 2009; Levchenko *et al.*, 2003). Through this PDZ binding motif, Amot, AmotL1, and AmotL2 are able to directly bind to PDZ-3, -1 and -2 domains of polarity protein Patj/Mupp1 respectively (Sugihara-Mizuno *et al.*, 2007). Simultaneously, Patj/Mupp1 associates with a RhoA GEF binding protein Syx to mediate Syx transportation from cell junctions to the leading edge of migrating ECs. Thus Amots contribute to the recruitment of Syx for to localize RhoA activity (Ernkvist *et al.*, 2009). In addition, another cell polarity protein Par3 binds to the PDZ domains of AmotL2 (Bratt *et al.*, 2002; Mojallal *et al.*, 2014). This binding enabled AmotL2 to localize to the cell junction together with VE-/E-cadherin, which was essential for radial F-actin organization (Hultin *et al.*, 2017). The physical association of AmotL2 to E-cadherin in epithelial cells and VE-cadherin in ECs was found to be highly dependent on the N-terminal PPxY motif (220-307a.a. and 101-307a.a., respectively) determined by Co-IP of different AmotL2 constructs (Hultin *et al.*, 2017; Hildebrand *et al.*, 2017). Furthermore, the shorter isoform p80 Amot has been identified as an interacting partner of mesenchymal junctional cadherin-Cdh11 (or osteoblast-cadherin) to promote epithelial cell migration (Ortiz *et al.*, 2015).

MAGI-1 is another crucial component that forms a complex with E-cadherin and α-catenin during cell junction formation in epithelial MDCK cells (Dobrosotskaya and James, 2000; Hildebrand *et al.*, 2017). Additionally, MAG1-1 influences actin cytoskeleton dynamics by being physically bound to the actin bundling proteins synaptopodin and α-actinin-4. Interestingly, AmotL2 was once given the name MAGI-1-associated coiled-coil tight junction protein, since it was identified from a glomerular cDNA library screening for MAG1-1 binding partners (Patrie, 2005). In the same year, another independent experiment confirmed the association between p130 Amot and MAGI-1b in CHO cells (Bratt *et al.*, 2005). In addition to stabilize junctional proteins, the N-terminal domain of p130 Amot transfection in MAE cells induced actin fiber formation that affected EC shape and motility (Ernkvist *et al.*, 2006), suggesting that Amot proteins might organize actin filaments and connect them to the cell junctions.

Epithelial or endothelial sheet/tube formations are the initial and fundamental processes of multicellular tissue construction and organogenesis, which requires the precise integration and regulation of multiple cellular events, such as apical-basal membrane polarity, intercellular junction and the organization and control of cell geometry by the cytoskeleton. Amot proteins are of interest because they localize membrane receptors with polarity protein complexes and the cytoskeleton, as well as regulate the Hippo pathway. Therefore these scaffolds integrate essential morphogenetic cues to control cell shape, cell polarity and the proliferation of multicellular tissues.

Taken together, those findings indicate that Amot proteins are able to integrate various structural proteins to influence cell morphology and function in vascular biology.

2 AIMS OF THIS THESIS

All three members of this family—Amot, AmotL1, and AmotL2—are scaffold proteins; they associate membrane receptors with polarity proteins and the cellular cytoskeleton. In this thesis, we have examined genetic mouse models to understand how these protein complexes are involved in vascular development, homeostasis and disease. We propose that the main role of the Amot family is to transmit mechanical cues from the environment to the cellular cytoskeleton.

To this end, my colleagues and I, have addressed the following specific aims:

Paper I

To study the role of Amot in transmitting mechanical force in migrating ECs.

Paper II

To experimentally test the "integrin switch" theory and develop a probe that can specifically detect strained Fn upon enhanced micro-environmental mechanics.

Paper III

To investigate the function of AmotL1 in vascular remodeling during angiogenesis and the underlying mechanisms of the interaction between ECs and pericytes required for vessel integrity.

Paper IV

To investigate the role of the VE-cadherin/AmotL2 mechanotransductory complex in suppressing inflammation in adult arteries.

3 METHODS AND MATERIALS

3.1 Mouse models

Animal models that can truly recapitulate developmental and pathological processes are extremely important in translational research and drug development. Among a number of organisms studied, mice have stood out as the most widely used model, despite differences in metabolic rate, diets, microbiomes and other aspects (Perlman, 2016). The irreplaceable advantages of mice models include high genetic homologies (85% on average) with humans, ease of genetic manipulation, relatively cheap maintenance, time-efficiency for high throughput studies et cetera (Vandamme, 2014; Waterston *et al.*, 2002; Rosenthal and Brown, 2007).

In this thesis, we have employed several mouse models and we are committed to the principles of the 3Rs (replacement, reduction and refinement) in experiment design. Ethical permits for Papers I, III and IV were obtained from the North Stockholm Animal Ethical Committee and the ethical permit for Paper II was granted by the Georgia Tech Institutional Animal Care and Use Committee (IACUC).

3.1.1 Conditional knockout mouse models (Papers I, III, and IV)

According to our aims to delineate the function of Amot proteins in vasculature *in vivo*, we established three endothelium-specific knockout mouse strain in the background of C57BL/6J based on a Cre-Lox recombination system (Sauer, 1987) (Monvoisin *et al.*, 2006).

3.1.1.1 amot^{flox}-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP mice *(amot^{ec-}* mice*)*

Amot, as the target gene, was flanked by two loxP sites for Cre-recombinase to bind. Specificity of the endothelium deletion was accomplished by control of Cre expression under Cdh5(PAC)(VE-cadherin) promoter. To gain control of Cre activity, sequences from the Cre gene as well as sequences encoding a mutant form-ER^{T2} of estrogen receptor binding domain were fused (Danielian *et al.*, 1993; Metzger and Chambon, 2001; Feil *et al.*, 1997; Leone *et al.*, 2003). Once tamoxifen is injected, the Cre protein gains freedom by dislocating from the fusion protein, in order to enter nucleus and initiate "prune" activity. In addition, to evaluate the activation of Cre-recombinase, we introduced the ROSA26-EYFP (enhanced YFP) reporter into *amot^{flox}*-Cdh5(PAC)^{CreERT2} mice. When Cre-recombinase targets the loxP sites YFP is expressed, which allows the efficacy of Cre-recombination on individual cells to be monitored by YFP immunostaining (Srinivas *et al.*, 2001).

Amot^{ec+} and *amot^{ec-}* are the abbreviations to refer to *amot*^{wt}-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP and *amot*^{flox}-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP mice, respectively. It is worthy to note that *amot gene* is located in the X chromosome, and we therefore use *amot^{ec+}* and *amot^{ec-}* for female mice to avoid confusion.

3.1.1.2 amotl1$^{flox/flox}$-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP mice *(amotl1$^{ec-/ec-}$* mice*)*

In the same fashion, we established a mouse model for the AmotL1 endothelium-specific KO mouse model. *Amotl1$^{ec+/ec+}$* and *amotl1$^{ec-/ec-}$* are the abbreviations for *amotl1$^{wt/wt}$*-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP and *amotl1$^{flox/flox}$*-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP mice, respectively.

3.1.1.3 amotl2$^{flox/flox}$-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP mice *(amotl2$^{ec-/ec-}$* mice*)*

Amotl2$^{ec+/ec+}$ and *amotl2$^{ec-/ec-}$* are the abbreviations for *amotl2$^{wt/wt}$*-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP and *amotl2$^{flox/flox}$*-Cdh5(PAC)^{CreERT2}-ROSA26-EYFP mice, respectively.

3.1.2 Mouse model of retinal angiogenesis (Papers I and III)

The mouse retina permits the study of angiogenesis in a natural context *in vivo* with several advantages that the other *in vitro* and *in vivo* models lack (Stahl *et al.*, 2010; Uemura *et al.*, 2006).

- Hierachical vascular networks forming only after birth allow us to observe diverse processes, including angiogenesis (tip cells sprouting), capilliary maturation, artery and vein differentiation et cetera.
- Laminar superfical blood vessel patterning is reproducible and accessible for monitoring and analysis.
- Mural cell, astrocyte and other cell types involved in angiognesis are well characterized.

As opposed to human babies, the retinas of new born mouse pups are avascular (Gyllensten and Hellstrom, 1954). Vessels start to expand from the central optic nerve immediately after birth and reach the periphery of the retina at P8. During the expansion of the vascular network migrating EC tightly followed the guidance of network of astrocytes (Dorrell, Aguilar and Friedlander, 2002). Proceeding the ECs, astrocyte begins to emerge from the optic nerve around E19 (embryonic day 19), and ECs therefore migrate on the pre-paved astrocytic template after birth (Dorrell and Friedlander, 2006). The developing retinal superficial vasculature is illustrated at characteristic time points postnatal day 2 (P2), P4 and P6 in ***Figure 10***.

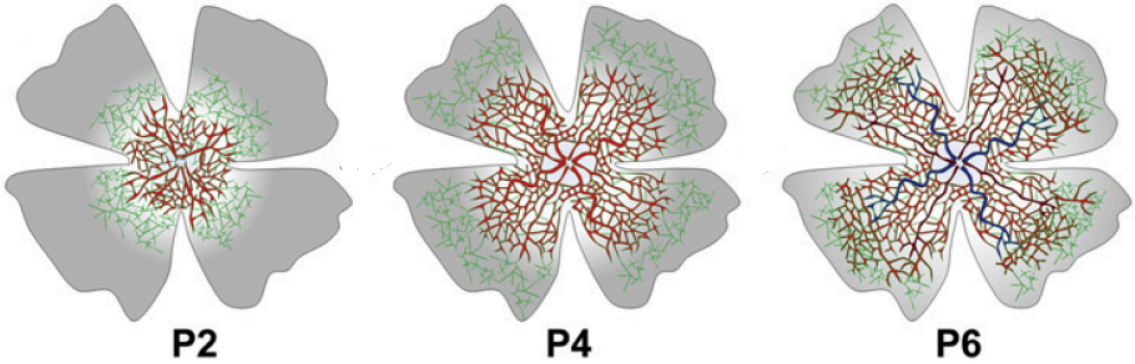


Figure 10. Schematic of postnatal vascular development in mouse retina. *During the first week, the superficial vascular plexus extends radially from the optic nerve. The time course of blood vessel growth in postnatal retinas was exemplified at P2, P4 and P6. The non-remodeled vascular plexus is shown in red, arteries in red, veins in blue and the Fn-expressing astrocyte network in green. Adapted from (Tata, Ruhrberg and Fantin, 2015). Reprinted with permission from the publisher.*

To induce Amot recombination and ablation, mice were treated with tamoxifen (400 μg/mouse/day) by intraperitoneal injections from P1 to P3. Normally, the Cre-recombinase efficiency was above 90%, tested by YFP immunostaining in our system. A lower dosage was also applied with the intention of decreasing recombination frequency to facilitate competitive studies of *amot*$^{ec+}$ and *amot*$^{ec-}$ cells for tip cell positioning. Retinas were harvested at P6 for retinal angiogenesis and subsequently analyzed according to the protocol by Pitulescu and colleagues (Pitulescu *et al.*, 2010). To ablate Amot in adult mice, tamoxifen (2 mg/mouse/day) was administrated for five days in a row, and the retinas was harvested seven days later.

3.1.3 Tumor transplantation mouse model (Papers I and III)

We studied tumor angiogenesis using the tumor with Lewis Lung Carcinoma (LLC) transplantation model. The LLC cells were originally derived from a C57BL/6 mouse and were thus syngeneic with our genetic mouse models. The LLC model has been extensively used for tumor angiogenesis studies due to its rapid growth with hyperneovascularization induced by pro-angiogenic factors released form the tumor cells.

Mice at six weeks of age were injected by tamoxifen to activate *gene* ablation. LLC cells (0.5×10^6/mouse) were injected subcutaneously on the last day of tamoxifen injection. Tumor growth was manually inspected twice per week, and tumor volumes were calculated. Starting from two weeks after the first injection, the same dosage of tamoxifen was injected into the tumor-bearing mice for five days. When tumors reached 10 mm in diameter, the mice were sacrificed according to the ethical permit.

3.1.4 MMTV-PyMT transgenic mouse model (Papers I and III)

The MMTV-PyMT transgenic mouse model is widely used to study mammary tumor progression and metastasis. In the MMTV-PyMT model, mammary gland–specific expression of the PyMT (polyoma middle-T) oncogene driven by the upstream mouse mammary tumor virus (MMTV) results in the rapid development of multifocal mammary adenocarcinoma occurring at around the age of 8–9 weeks (Lin *et al.*, 2003).

We crossed our conditional knockout mice (*amot*$^{ec-}$ and *amotl1*$^{ec-/ec-}$*)* mice into the MMTV-PyMT background and induced *gene* endothelial specific depletion at week four by a tamoxifen injection five days (2 mg/mouse/day) prior to the occurrence of breast tumors.

3.1.5 Mouse bleomycin model of pulmonary fibrosis (Paper II)

Idiopathic pulmonary fibrosis is a chronic disease that leads to death, and the bleomycin animal model is one of the best mouse models available to study the pathogenesis and test a potential drug (Mouratis and Aidinis, 2011). In addition, this model has been characterized by high reproducibility and a variety of administration routes. Bleomycin can induce DNA breaks that lead to lung injury and fibrosis (William Lown and Sim, 1977) and acute lung injury can be observed after seven days followed by a 3-4 week fibrotic stage, and then the pathological situation can return to normal with minimal fibrosis (Chung *et al.*, 2003).

Female mice at six weeks of age were anesthetized using a ketamine/xylazine/acepromazine cocktail, and injected with bleomycin sulfate at a dose of 2 U/kg (50 µL with 10 µL of air bolus) through intratracheal injections. Mice were placed on a warm pad until they recovered and lung tissues were harvested

3.1.6 Mouse model of aortic function (Papers I and III)

An AAA is a focal dilation of the aorta and may rupture, resulting in extensive bleeding into the abdominal cavity. The prevalence of AAA increases with age in humans. To mimic the human condition as closely as possible, our studies recruit mice (both males and females) aged between 6 and 8 months (the alleged lifespan of our mouse strain is 1.5 years). Four weeks after we induce gene knockout by five days of standard tamoxifen injections, we harvest the aorta, vena cava, bladder and other organs for analysis. The dissection and perfusion protocol follows (Robbins *et al.*, 2014).

Despite the primary advantages of the mouse model in our study on vascular inflammation, including the low cost, ease of breeding and genetic manipulation and rapid development of atherosclerosis, some characterized vascular events cannot be fully recapitulated in the mice (Getz and Reardon, 2012). For instance, the sites of atherosclerosis in mice usually occur in the aortic arch and innominate artery, rather than the carotids and coronary arteries in humans. This is a reminder of the problems of recapitulating human diseases in mouse models.

3.2 Human materials

To corroborate the data from our mouse models presented above, we also analyzed human patient materials, primarily using immunohistochemical (IHC) stainings and RNA expression analysis.

3.2.1 Human Protein Atlas (Paper I)

The Human Protein Atlas is a Swedish-based program initiated in 2003 and aiming to map all the human proteins in cell, tissue, and organs (*http://www.proteinatlas.org*). Various omics technologies have been included for the exploration of the human proteomes, for example, imnnunostaining imaging and mass spectrometry-based proteomics (Uhlen *et al.*, 2015).

3.2.2 Human aortic aneurysm samples (Paper IV)

Human AAA samples were obtained from vascular surgeries in Karolinska Hospital, Media and adventitia parts of the AAA samples without any thrombosis attachment were analyzed by GeneChip™ Human Transcriptome Array 2.0.

Proper ethic permits were granted by *Regionala etikprövningsnämnden i Stockholm*.

3.2.3 Human primary cell line (Paper IV)

For studies of AmotL2's function in aortic and venous endothelium *in vitro*, we purchased primary cell lines of the Human Aortic Endothelial Cell (HAoEC) and Human Umbilical Vein Endothelial Cell (HUVEC), respectively. As stated previously, the incidence of AAA is higher in males over middle age. Therefore the HAoEC cell batch we used in Paper IV was from a Caucasian male donor at age 55.

To maintain the proper characters of primary cells, we performed experiments only on those cells at low passages (less than five) and cultured the cells under standard guidelines (Geraghty *et al.*, 2014).

4 RESULTS AND DISCUSSION

4.1 The Amot/integrin protein complex transmits mechanical forces required for vascular expansion. (Paper I)

4.1.1 Amot is primarily expressed in developing blood vessels.

Amot has been reported to express preferentially during physiological angiogenesis of zebrafish and mouse (Aase *et al.*, 2007). However, other publications have indicated Amot as a regulator of the Hippo pathway in epithelium *in vitro* to affect tumor progression (Lv *et al.*, 2017). To clarify the discrepancy, we mapped the expression pattern of Amot in normal and cancerous human tissues using immunoaffinity-purified Amot antibodies with high specificity (Levchenko *et al.*, 2003). Amot positivity was restricted to angiogenic sites, such as blood vessels of the third-trimester placenta. Furthermore, Amot was barely detectable in areas outside the blood vessels in adult tissue such as brain and breast. Consistently, up-regulation of Amot in tumor vessels and stroma was observed in human cancers of different origins, whereas the surrounding tumor cells were negative. We therefore have concluded that Amot primarily functions in angiogenic blood vessels during tissue remodeling after birth.

4.1.2 Amot is required for both retinal and tumor angiogenesis.

Using our endothelium-specific knockout mice, we inactivated *amot* in ECs by tamoxifen injections during the first three postnatal days. A significant non-symmetrical and partially collapsed vessel expansion could be observed in $amot^{ec-}$ retina of P6 (***Figure 11***). Confocal microscopy analysis revealed a significant decrease in the number of tip cells, as well as reduction in filopodial extensions. Interestingly, no blood vessel aberrations were detected when Amot was deleted in adult mice, suggesting that Amot only functions in developing blood vessels.

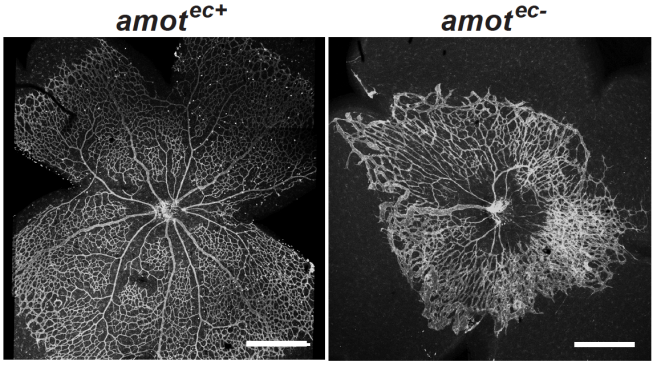


Figure 11. Poor expansion of blood vessel network in $amot^{ec-}$ mouse retina. *The blood vessel network was visualized by IB4 staining.*

To investigate whether the impaired vascular expansion is caused by a defect in tip cell migration rather than capillary development, we decreased the doses of tamoxifen injection, resulting in lower recombination frequencies (YFP positivity). When the recombination frequencies are lower than 85%, $amot^{ec-}$ EC showed a decreased ability to localize in tip cell

positions but no obvious ratio change in capillaries, which indicated its disadvantages in leading the migrational front.

As we could detect the up-regulation of Amot in blood vessels of human tumors, we further investigated whether Amot also played a role in tumor angiogenesis. Here we employed two tumor models for pathological angiogenesis: Lewis Lung Carcinoma (LLC) transplantation and the transgenic mouse model of breast cancer MMTV-PyMT. First, LLC tumors growing in an Amot EC–free environment were significantly smaller as assessed by tumor weight, than $amot^{ec+}$ mice. Immunostaining sections from resected tumors showed a marked decrease in vascular density and an increased percentage of necrosis. In the second transgenic breast tumor model, we induced *amot* endothelial specific depletion at week four. Normally, multiple breast tumors occur at around 8–9 weeks of age in $amot^{ec+}$ mice; however, an analysis of early onset tumor occurrence displayed an average of six weeks longer latency in $amot^{ec-}$ mice, confirming the protective effect of *amot* deletion on tumor incidence by inhibiting angiogenesis.

Our previously studies have reported a DNA vaccination against Amot, which blocked angiogenesis, thereby preventing growth of a transplanted tumor *in vivo* (Holmgren *et al.*, 2006). In addition, an Amot antibody (Bo6-scFv specific for human and mouse p80 Amot) has been testified for its anti-angiogenic effect in mice (Levchenko *et al.*, 2008). In conclusion, we propose that Amot is a potential therapeutic target to inhibit both physio- and pathological angiogenesis. However, to make the targeting antibody suitable for long-term treatment *in vivo*, more work must be done to prolong the half-life in circulation.

4.1.3 Amot relays force between EC and ECM *in vivo* and *in vitro*.

Early work by Ingber and Folkman showed that mechanical interactions between ECs and ECM regulated capillary development *in vitro* (Ingber and Folkman, 1989). This led to research that showed that ECM properties, such as matrix stiffness, controlled cell shape and function by modulating the cytoskeletal network (Ingber *et al.*, 1995). A hypothesis was proposed that the lack of contractile force between endothelial tip cell and ECM causes, at least partially, the collapsed vasculature in the absence of Amot. Here we provide three approaches to show that Amot is essential for the transmission of force between the Fn component of ECM and the cytoskeleton of migrating ECs.

1. EC-specific knockour of Amot led to changes in Fn fibrillogenesis *in vivo* as analysed by enhanced resolution confocal microscopy. That is, Fn fibrils were stretched and aligned with tip cell filopodia in $amot^{ec+}$retina, whereas Fn was localized to punctate and shorten fibrils in $amot^{ec-}$, implying less local extension.
2. We exploited H5-scFv targeting a conformation change of Fn when exposed to mechanical strain, thus unmasking a cryptic epitope (more details in Paper II). Amot deficient cells had a lower ratio of strained vs pan-Fn in the adjacent matrix.

3. Direct measurements of force using TFM on an Fn-coated matrix *in vitro* supported the notion as well. Amot siRNA–depleted cells exhibited a significant decrease in the force exerted on the Fn-coated polyacrylamide gel.

4.1.4 Amot is a novel component of the integrin adhesome.

To investigate the molecular mechanisms involved in the Amot-mediated force transmission, we performed RNA sequencing analysis (RNA-seq) of control and Amot KD cells and Mass Spectrometry (MS) analysis of Amot-associated proteins in BAE cells. To focus on the cell-matrix interaction, the cells used for *in vitro* study were cultured at 40% confluent. The omics analyses unveiled an enrichment of genes that positively regulate the "focal adhesion" and "ECM-receptor interaction" KEGG pathways, and several integrin subtypes were recognized from the protein list, including β3, α5, and β1. We verified these data by immunoprecipitation analysis using either Amot- or integrin-specific antibodies. Interestingly, Amot siRNA depletion resulted in less actin associated with primarily the αvβ3-integrins. Consistently, a recent study reported that endothelial tip cell migration during angiogenesis involves mechanical force to unfold Fn with αvβ3-integrin binding sites in ECM (Li *et al.*, 2017).

To further identify the subcellular localization of Amot, we performed an *in situ* proximity ligation assay (PLA) to analyze the co-localization of Amot with the focal adhesion protein vinculin. The PLA positive dots overlapping with actin filaments indicated a close proximity (proteins within 40 nm) of Amot and vinculin in MS1 cells. Importantly, this signal was essentially abrogated by Amot siRNA depletion, but could be rescued by re-expression of Amot using a mouse p130-Amot-GFP plasmid transfection. The same results were obtained from HEK 293 cells, a cell line that we earlier reported expressed high levels of p130 Amot. In all, our data show that Amot is a novel component of the integrin adhesome in ECs.

The integrin adhesome has been extensively studied and over 200 proteins have been implicated in the cell-ECM interaction (Horton and Humphries, 2016). In 1994, αvβ3 became the first integrin proposed to be high expressed in angiogenic ECs (Brooks *et al.*, 1994). Since then, extensive studies has focused on inhibiting αvβ3 to suppress tumor angiogenesis without affecting quiescent ECs. Genetic deletion before a tumor challenge transiently suppressed tumor angiogenesis; however, the effect on growing tumors was minimal (Steri *et al.*, 2014). Certainly, there are more subtypes of integrins and multiple tension-sensitive binding partners involved in blood vessel growth in tumors. Further insight on how mechanical force transduced may shed light on better strategies to target those pathways.

The role of Amot appears to be the formation of actin filaments. Previous work has shown that Amot binds and promotes F-actin formation, which regulates the activity of the YAP transcription factor (Ernkvist *et al.*, 2006; Mana-Capelli *et al.*, 2014). The interaction with the cytoskeleton is at least partially mediated by the Rho-GEF protein PLEKHG1/Syx, which binds Amot and promotes EC migration by governing RhoA GTPase activity (Ernkvist *et al.*, 2009; Garnaas *et al.*, 2008).

Taken together, we proposed a mechanism of endothelial tip cell migration on ECM enabled by mechanical force transition unit-Amot/Integrin-associated adhesive protein complex in charge of relaying force between ECs and the ECM (***Figure 12***).

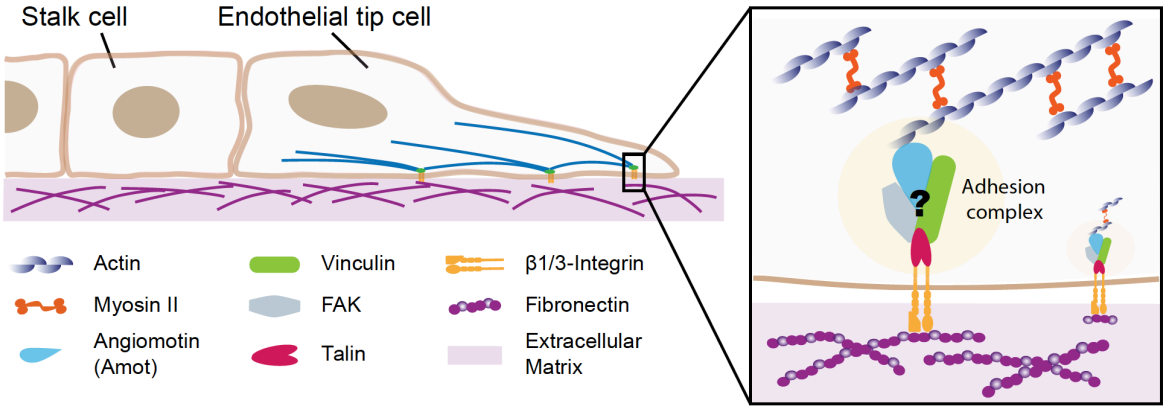


Figure 12. Amot as a component of an integrin-associated adhesion complex. *Full aortae of amotl2$^{ec+/ec+}$ and amotl2$^{ec-/ec-}$ were anatomically isolated and presented in the left image. A wild-type piece of aorta was framed in the orange box and AAA in the blue box. The images with whole-mount staining of TOPRO (blue), F-actin (red) and VE-cadherin (green) were shown on the right.*

4.2 Detection of an Integrin-binding mechanoswitch within fibronectin during tissue formation and fibrosis. (Paper II)

4.2.1 Fn strain drives differential integrin affinity.

Krammer and his team have forwarded the theory of a "switch" of the integrin binding profile of Fn triggered by mechanical forces (Krammer *et al.*, 2002). To verify this theory with experimental evidence, we used two pre-engineered Fn fragments spanning the ninth and 10th type III repeats of Fn to test whether integrins have preferential affinity (Brown *et al.*, 2015; Martino *et al.*, 2009). Simply speaking, FnIII9*10 is the native and stabilized fragment, whereas FnIII9-4G-10 has an approximate 0.9 nm separation between the PHSRN and RGG sites, therefore representing a strained conformation of the Fn fragment. By SPR experiments and the IF staining of integrins on cells culture on those Fn fragments, we are the first to show that α5β1-integrin prefers to bind the native FnIII9*10, and the affinity were decreased on the mechanically perturbed FnIII9-4G-10 fragments. This switch was not observed on αvβ3-integrin, since αvβ3 has similar binding affinity to FnIII9-4G-10 as well. A similar high affinity of α5β1-integrin engagement on strained Fn has been articulated earlier, using a designed Fn-mimetic peptide (Craig *et al.*, 2008). These observations together lead us to develop a promising probe to further confirm the switch theory.

4.2.2 H5 scFv was developed to detect conformation change of strained Fn.

To generate a probe targeting the force-sensitive conformational change in the integrin-binding domain of Fn, we used FnIII9*10- and FnIII9-4G-10-predicted Fn fragments as

template models. In addition to the extended distance between PHSRN and RGD due to the 4G insert in FnIII9-4G-10, the stability also differs in the flexible 4G regions. This flexibility is of importance to recapitulate the sensitivity of FnIII repeats to force-induced unfolding (Krammer *et al.*, 2002; Li *et al.*, 2005). By performing phage display panning and selection based on scFv antibody libraries on those Fn fragments (de Wildt *et al.*, 2000; Lee *et al.*, 2007), Clone H5 stood out among the 40 clones as displaying a high affinity against the extended FnIII9-4G-10, but not to the stabilized conformation of FnIII9*10.

To validate H5's recognition of FnIII9, we manually deposited an Fn fiber pre-patterned and stretched PDMS (polydimethylsiloxane) membrane. With the increase of strain manipulated by the system, H5 binding displayed a switch-like behavior: The H5 signal sharply increased at the extension ratio of 1.4–1.5 and plateaued with further stretching (***Figure 13***). Similarly, on another independent system of elastic PDMS membrane assembling decellularized Fn-rich ECM, H5 staining was able to specifically recognize strained Fn over relaxed Fn ECMs, both globally and locally. The intensity of H5 staining was strongly regulated by the contractile state of the cells, as induced by TGFβ (contractile agonist) and inhibited by blebbistatin (inhibitor).

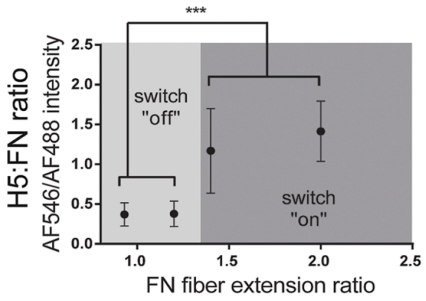


Figure 13. H5 scFv is capable of targeting conformational change in Fn's integrin-binding domain. *The transition of the "switch" from low to high binding affinity occurs at the extension ratio of 1.25–1.5.*

4.2.3 H5 probe highlights strained tissues in *ex vivo* models.

In addition to validating H5 *in vitro*, we tested H5 in two well-established mouse model systems: pulmonary fibrosis and postnatal retinal angiogenesis (described in *Methods and Materials*). Bleomycin treatment induced severe fibrosis two weeks after administration. Three to four weeks later, fibrosis began to resolve and almost returned to a normal state at week 10. Radiometric images analysis of H5/pan-Fn staining exposed a positive correlation between H5 intensity and the severity of fibrosis, which suggested that H5 is able to distinguish the conformational states of Fn within the native tissue.

In another mouse retinal angiogenesis model, the vascular network starts to expand at birth (Pitulescu *et al.*, 2010). The migrational endothelial tip cells are guided by an Fn template pre-deposited by astrocytes (Jiang *et al.*, 1994). H5/pan-Fn ratios were significantly higher in areas where the filopodia of tip cells interact with elongated Fn fibrils, compared to established mature capillaries. These combined results proposed a mechanism whereby forces that are generated from endothelial tip cells unfold Fn to expose αvβ3-binding sites within the ECM influencing angiogenesis. Interestingly, Paper I verifies that Amot relays forces between tip cells and Fn fibrils through integrin-associated adhesome. αvβ3-integrin shows

more dependency on Amot than α5β1-integrin with respect, regarding to connecting the cytoskeleton to exert a contractile force. This finding is further supported by research conducted by (Li *et al.*, 2017), which emphasizes that α3/α5β1- and αvβ3- integrin stimulation may be harnessed to have the opposite influence during reparative angiogenesis. Overall, the data suggested a potential integrin binding specificity in developing tissues.

Finally, we have provided an experimental demonstration of switched integrin binding to Fn with force-sensitive conformational changes. Furthermore, an H5 probe shows a convincing ability to detect a stretched conformation of Fn in native tissues, which increases the possibility of using H5 as a paradigm for the imaging and diagnosis of fibrotic tissues.

4.3 AmotL1 is a novel component of the N-cadherin complex affecting endothelial/pericyte interaction in normal and tumor angiogenesis. (Paper III)

4.3.1 AmotL1 deficiency affects vessel formation in mouse retina and tumor.

The aim of this paper was to study the role of AmotL1 in vascular development. To this end, we analyzed the effects of *amotl1* inactivation during postnatal vessel expansion in mouse retina using EC-specific KO models as previously described in Paper I. The phenotype differed significantly from that of the *amot* ablated retina. The *amotl1*$^{ec-/ec-}$ vascular network exhibited a significant reduction in vascular density and a decreased amount of branching in the periphery as well as in the central retina. Besides the ECs, other cell types also contribute to the forming function of the circulatory network. Microglial cells and astrocytes did not show any obvious macroscopic differences in appearance. However the whole-mount immunostaining showed that the area of pericyte coverage decreased and the cells "bulged out" from the retinal vessels (depicted in ***Figure 14***).

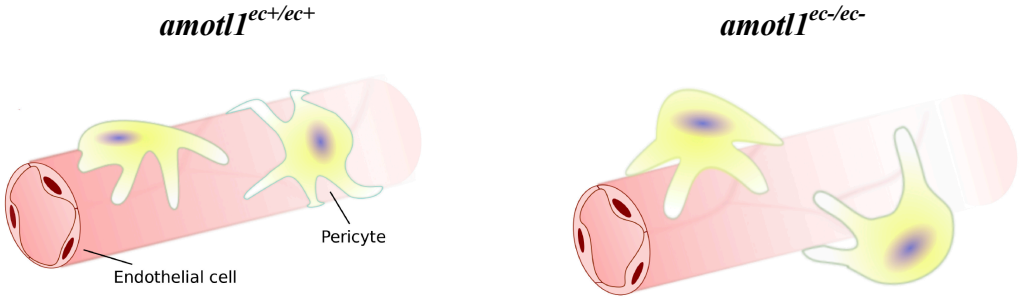


Figure 14. Schematic illustrating poor pericyte coverage in an amotl1$^{ec-/ec-}$ vessel. *Pericyte bulges out with AmotL1 absence in the endothelium as a non-autonomous effect.*

In addition to physiological vascular development, we assessed the role of AmotL1 in the vasculature of the LLC transplantation and MMTV-PyMT mouse tumor models, as described previously. Tumors growing in the absence of AmotL1 in endothelium had a significant

increase in vessel diameter. A prolonged period of tumor latency in *amotl1*$^{ec-/ec-}$ mice was suggested to be attributed to the lower functionality of tumor blood vessels.

Extensive studies have shown that endothelial-derived platelet-derived growth factor subunit B (PDGFB) is crucial for pericyte recruitment in newly formed vessels (Armulik, Genove and Betsholtz, 2011). Genetic ablation of PDGFB changes the retinal vascular density and enlarges the tumor blood vessel diameter (Abramsson, Lindblom and Betsholtz, 2003), a finding quite similar to our discoveries during normal and tumor blood vessel development.

How does AmotL1 KO in endothelium cause vessel dilation and generate a defect of contacting pericyte coverage in a non- autonomous fashion?

4.3.2 AmotL1 belongs to N-cadherin associated adhesive junction between EC and pericyte.

ECs and pericytes are tightly connected in functional mature blood vessels. The two distinct cell types communicate by paracrine biochemical factors as well as direct physical contact in cell–cell adhesions mediated by N-cadherin (Gerhardt, Wolburg and Redies, 2000). Endothelial cells also express N-cadherin that is, in contrast to VE-cadherin, not endothelial specific, as it is expressed in multiple-types of mesenchymal cells, such as pericytes (Cavallaro, Liebner and Dejana, 2006). Therefore N-cadherin is a possible binding partner for AmotL1. However, no binding evidence could be detected between AmotL1 and N-cadherin by Co-IP analysis. Previous reports have suggested that N-cadherin junctional localization is governed by VE-cadherin expression (Navarro, Ruco and Dejana, 1998; Hordijk *et al.*, 1999; Giampietro *et al.*, 2012), and *in vitro*, N-cadherin has a dispersed distribution on the cellular membrane, however it changed location to adherent junctions in VE-cadherin depleted cells (Salomon *et al.*, 1992). As expected, using cells either treated by VE-cadherin siRNA to knock down AmotL1, and purified from VE-cadherin$^{ec-/ec-}$mice, AmotL1 was detectable from N-cadherin immunoprecipitate, but not Amot or AmotL2. This is the first time that AmotL1 was shown to be a part of the N-cadherin adhesion complex when N-cadherin is recruited to cell junctions

N-cadherin is commonly expressed in mesenchymal cells including vascular mural cells, such as SMC (smooth muscle cell). Ji-Hye Paik and colleagues have reported that blocking N-cadherin binding in *in vitro* co-cultures abrogated the interaction of ECs and pericytes (Paik *et al.*, 2004). The results are also supported by *in vivo* data that N-cadherin is required for the blood vessel stabilization by regulating the interaction of ECs and pericytes (Gerhardt, Wolburg and Redies, 2000). Furthermore, the genetic inactivation of N-cadherin resulted in impaired pericyte coverage of endothelial sprouts *in vitro* (Tillet *et al.*, 2005), similar to our findings with AmotL1 KO.

These data raised an interesting question: The EMT (epithelial-mesenchymal transition) involves the down-regulation of E-cadherin and the up-regulation of N-cadherin, which assists the adhesion to ECs and thus extravasation and metastasis (Wells *et al.*, 2011). This leads to the question of whether AmotL1 is involved in tumor cell migration and spreading as

well. A recent study has shown that AmotL1 is up-regulated and promotes invasion in breast cancer (Couderc *et al.*, 2016). Moreover, protein analysis on tumor samples from MMTV-PyMT mice has manifested a high level of AmotL1 expression. Further studies may shed light on whether the tumor–endothelial interactions are dependent on mechanical forces relayed by AmotL1.

In summary, AmotL1 is identified as a specific partner of N-cadherin in both ECs and pericytes and the tensional balance maintained by and proper endothelial/pericytes interaction is important for vascular network organization.

4.4 The VE-cadherin/AmotL2 mechanosensory pathway suppresses aortic inflammation and the formation of abdominal aortic aneurysms. (Paper IV)

4.4.1 AmotL2 is essential for aortic morphology in response to flow.

Previous data has shown that AmotL2 is essential for aortic lumen expansion during embryonic development in both mouse and zebrafish models (Hultin *et al.*, 2014). To explore AmotL2 functions in the aorta of adult mice, we first mapped the AmotL2 expression pattern in the largest vessels: the descending aorta (DA) and the inferior vena cava (IVC). The immunostaining with AmotL2 revealed that AmoL2 was primarily expressed in the aortic endothelium rather than the IVC. This preference was also observed in major retinal arterials in adult mice.

The ECs of the DA tended to be aligned with the direction of blood circulation and contained radial actin filaments that were connected to the cellular junctions. By contrast, the ECs of the IVC exhibited a more rounded cellular shape with no or few detectable radial actin filaments. Using an inducible AmotL2 knockout mouse model to abrogate AmotL2 in adult mice, we observed a loss of radial actin filaments and a rounder cell shape in the ECs of DA one month after AmotL2 inactivation. However, a similar change was not detected in IVC endothelium. This artery-specific phenotype was also observed in the arterial ECs of other organs, such as the urinary bladder. This phenomenon could also be recapitulated in *in vitro* flow experiments. When HAoEC cells were exposed to 14dyn/cm^2 for 48 hours in a flow chamber (Ibidi system), the depletion of AmotL2 by a Lentivirus-based approach resulted in failure to elongate and align with the direction of flow as well as the absence of radial actin firmaments crossing cells.

Remarkably, we were able to induce an artery-like phenotype in venous HUVECs simply by re-expressing AmotL2. The localization of AmotL2 could be observed at cell–cell junctions in Lenti-AmotL2 transfected cells, which overlapped with VE-cadherin by both immunostaining and PLA. Interestingly, AmotL2 over-expression was sufficient to induce radial actin filament formation and to elongate and align cells even under static conditions.

VE-cadherin has been proposed to associate PECAM-1/VEGFR2 as a mechanosensory complex facilitating ECs in response to flow shear (Conway *et al.*, 2013b). Consistently, our data demonstrated that a VE-cadherin/AmotL2 junctional complex modulates mechanical tension across ECs, which is required for cell alignment in arterial ECs.

4.4.2 Inactivation of AmotL2 alters aortic nuclear shape and positioning.

The nucleus is the largest organelle of the cell, and its shape and positioning are determined by mechanical challenges from actin filaments to the nuclear lamina (Lele, Dickinson and Gundersen, 2018). As ECs are exposed to the hemodynamic drag by the blood flow, EC nuclei elongate and nuclei orient themselves relatively in the middle of the cell in response to flow. In *amotl2*$^{ec+/ec+}$ mice, endothelial nuclei of the DA were orientated in parallel with cell alignment in the direction of blood flow. However, in *amotl2*$^{ec-/ec-}$ ECs, the nuclei were rounded with irregular shapes and positioned close to the cell edge downstream of the direction of flow. These changes in nuclear shape and positioning were not observed in the IVC. Consistent results were obtained from *in vitro* experiments, in which nuclei from AmotL2-depleted HAoECs failed to be elongated and positioned near the center of cells under flow condition. These data indicated that the VE-cadherin/AmotL2/actin complex is required for proper nuclear positioning and alignment.

4.4.3 AmotL2 couples VE-cadherin to nuclear membrane through radial actin filaments.

Actin filaments are coupled to the nuclear membrane through the LINC complex, which consists of SUN/Nesprin proteins connecting to Lamin A/C of the nuclear lamina. The LINC proteins have also been demonstrated to relay mechanical signals between the cytoskeleton and the nucleus and thereby control nuclear positioning (Zhu, Antoku and Gundersen, 2017; Chang *et al.*, 2015; Gundersen and Worman, 2013a; Lee and Burke, 2018). Next, we investigated a possible connection between VE-cadherin, AmotL2, Actin, and the LINC complex. As we speculated, Lamin A/C, together with a few other molecules located in nuclear membranes, was identified from AmotL2 Co-IP in two endothelium cell lines (BAE and MS-1) through MS analysis. Notably, we verified this association *in vivo* IP from mouse lungs (which contain 30% ECs), which supported that AmotL2 binds with VE-cadherin, actin, and Lamin A/C. Furthermore, this association was proven to be dependent on myosin contraction and actin filaments, as both treatments with blebbistatin (the myosin II inhibitor) and cytochalasin D (the actin polymerization inhibitor) were able to inhibit the connection between AmotL2 and Lamin A/C. Additionally, IP of VE-cadherin confirmed that the association of VE-cadherin and Lamin A/C relies on AmotL2 as a link. Taken together, we proposed a novel mechanical pathway through which VE-cadherin/AmotL2 forms a complex that is connected to the nuclear LINC complex via actin filaments.

4.4.4 Deficiency of AmotL2 provokes vascular inflammation and aortic aneurysm.

The findings of such critical changes in *amotl2*$^{ec-/ec-}$ EC properties raised the question of whether impaired mechanotransduction of the EC could result in vascular inflammation or other severe consequences in such a high shear stress exerted from the blood flow. Due to the distinct properties of shear stress caused by the blood flow in the aortic arch (turbulent flow) and the DA (laminar flow), we separated the aortic arch and the DA into two distinct aortic tissues for further studies.

Whole mount immunostaining with CD45 (a general marker for leukocytes) antibodies in aortic arches in 45% of the *amotl2*$^{ec-/ec-}$ animals. The macrophage-like CD45^{+} cells resided physiologically in the inner curvature of the arches, however, the cells attached in the *amotl2*$^{ec-/ec-}$ arches were monocyte-like round cells, and some of them invaded the smooth muscle layers in intima. Due to the size difference between mice and humans, a mouse model has limitations in mimicking human situations; for example, atherosclerosis in humans usually occurs in carotids and coronary arterials, whereas in mice, the pathological change can be observed in the aortic arch (Getz and Reardon, 2012). Therefore, it is consistent with the clinical findings that initiation of atherosclerosis is characterized by monocytes infiltrating the arterial wall and thereafter differentiates to macrophages (Tsujikawa *et al.*, 2019).

Aortic inflammation commonly leads to aneurysm formation as inflammatory aortic aneurysms account for 5% to 10% of all AAA cases. Indeed, during aorta dissection under the stereomicroscope, we found that observable AAAs formed in 5 out of 45 *amotl2*$^{ec-/ec-}$ mice (25 males and 20 females), all of which were males (20% incidence), whereas no aneurysms were found in any of the 36 *amotl2*$^{ec+/ec+}$ mice (20 males and 16 females). The detected aneurysms occurred in the proximal of the renal artery branch of the DA. It is noteworthy that the actual AAA incidence in *amotl2*$^{ec-/ec-}$ mice might be largely underestimated due to their limited size in early developmental stages. Through IHC staining on cross-sections, media degradation and fibro-muscular hyperplasia were observed at the *amotl2*$^{ec-/ec-}$ aneurysm, and these pathological changes caused subsequent detrimental changes such as endothelial rupture and inflammatory invasion into peripheral renal arteries. The direct impacts of these changes on elastin were profound, as the elastic layer was disrupted and degraded at the sites where inflammatory cells invaded.

The mRNA expression level of immune-related genes by RT-qPCR revealed a significant increase of general inflammatory markers of Il6, Tnf, and Cd68 (macrophage marker) in both aortic arches and DAs, suggesting an activation of general and global immune response in *amotl2*$^{ec-/ec-}$ mice. To clarify which types of immunoresponse were induced, we examined chemokines such as Ccl2/MCP1 (monocyte chemoattractant protein 1), Ccl5, and Cxcl10 (NK cells), which are frequently up-regulated in experimental models of inflammatory diseases and thereby considered a hallmark for inflammatory cell infiltration (Aukrust *et al.*, 2008). Significant up-regulation of Ccl2 and a slight increase in Ccl5 and Cxcl10, which were

markers for monocyte, neutrophils, and NK cells penetration respectively, suggested a specific infiltration of monocytes in aorta, especially in DAs. By contrast, the T and B cell markers Cd4, Cd8, and Cd19 did not exhibit a significant difference on the mRNA level in *amotl2*$^{ec-/ec-}$ aortae. These data supported the conclusion that it was the innate rather than adaptive immune response that was provoked by AmotL2 deletion.

In summary, we propose that AmotL2 is essential for EC alignment and nuclear positioning in response to arterial shear stress. Inactivation of this pathway induces a pro-inflammatory immune response ultimately leading to the degradation of the aortic wall and resulting in the formation of an aortic aneurysm (***Figure 15***).

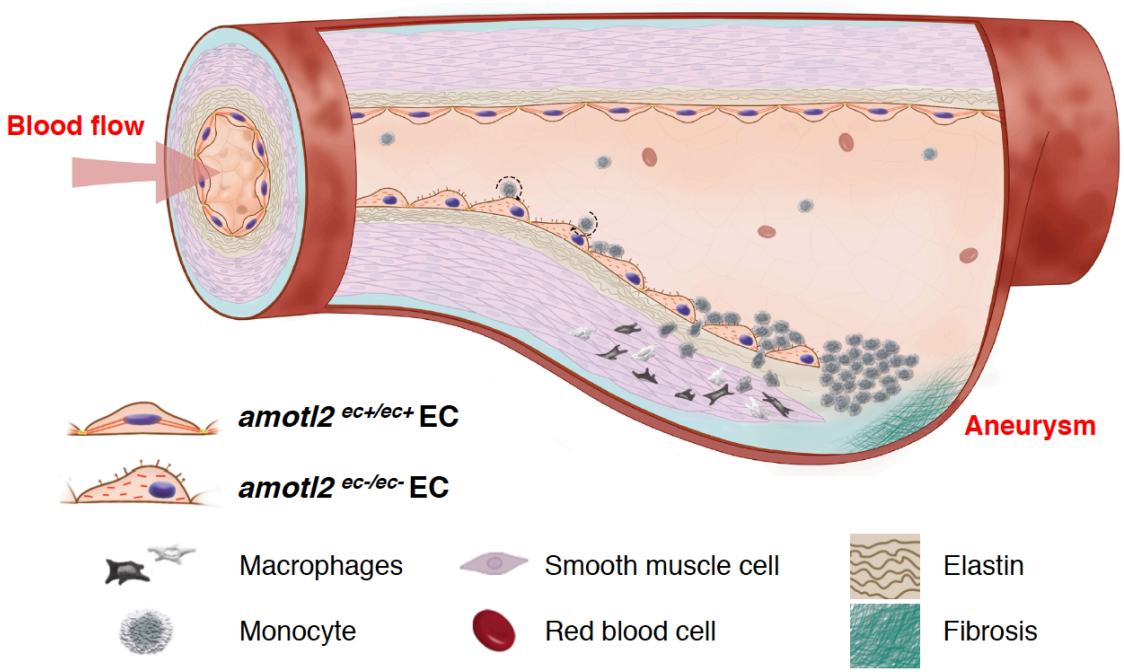


Figure 15. Hypothetical schematic of the formation of an AAA. *The step-by-step formation of an AAA includes EC activation, immune cell rolling and attachment, leukocyte extravasation, macrophage differentiation, thrombus formation, and finally, lumen dilation (aneurysm). Illustrated by Yu-Hsuan.*

5 CONCLUSIONS

Using a combination of experimental animal studies, molecular analysis, and biophysical technologies, my thesis highlights three mechanosensory pathways mediated by the Amot proteins. I provide evidence that the Amot protein family manipulates biomechanical complexes to orchestrate intrinsic and extrinsic forces driving vascular development.

More specifically,

- The Amot/Integrin protein complex at focal adhesion transmits mechanical forces required for both physiological and pathological vascular sprouting.
- The AmotL1/N-cadherin complex affects endothelial/pericyte attachment in normal and tumor angiogenesis.
- The AmotL2/VE-cadherin complex at endothelial cell-cell junction suppresses aortic inflammation and the formation of abdominal aortic aneurysms

Additionally, an integrin binding switch mediated by the Fn force-sensing domain has been newly identified. Moreover, a mechanosensitive scFv probe named H5 has been engineered to recognize and target the strained Fn that is subjected to enhanced forces in tissues.

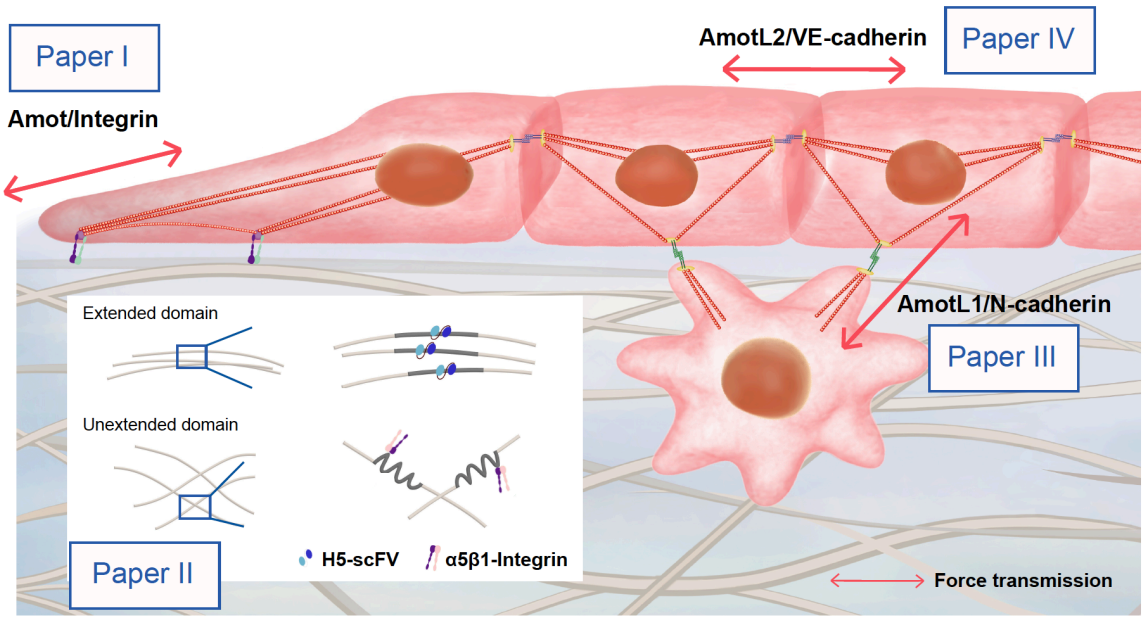


Figure 16. Ready to pull-force transmission during vascular development. *A schematic summarizes the findings in this thesis. Paper I: During vascular development, endothelial tip cells migrate by interacting with ECM through the Amot/integrin focal adhesive complex. Paper II: A separate image at the bottom left delineates α5β1 integrin switch between the FnIII9*10 (unextended) domain to the FnIII-4G-10 (extended) domain of Fn, as well as the specific binding of H5-scFv to extended Fn. Paper III: EC and pericyte form AmotL1/N-cadherin adhesive junction complexes. Paper IV: ECs connects to each other through an AmotL2/VE-cadherin junctional protein complex. Illustrated by Yu-Hsuan Hsu.*

6 FUTURE PERSPECTIVES

The field of mechanobiology is rapidly expanding and its implications for our understanding of human disease are becoming increasingly clear. The holistic view of mechanical forces and transduction pathways should be integrated into our mind, when we perceive molecular knowledge.

During the writing of this thesis I have begun to contemplate the future directions for translating existing data into clinical approaches and how to address the questions those findings have raised.

6.1 Mechanobiology development

The optimization of the engineering technologies concerning more feasible force indicators will be subject to further studies to develop new materialistic measurements. If the whole aortic endothelium surface could be mapped as a force landscape, we could directly visualize the lumen topography under the blood flow: points/area with altered force due to disturbed flow, where ECs are under high tension with a higher risk to be deformed et cetera. This would be extremely helpful in managing diseases with more precise intervention and treatment. Conwey and colleagues have developed FRET-based tension sensors for VE-cadherin and PECAM-1, and these enable the visualization of increased junctional tension in real time when the fluid shear stress is switched on (Conway *et al.*, 2013a). AmotL2/VE-cadherin, in my view, might also be a good pair of sensors due to AmotL2's preferential expression in arteries, where higher shear stress is applied.

With the probe H5 that we generated, combined with pan-Fn, we can delineate a force map in mouse fibrotic lung tissue and developing retinal vessels, and this might shed light on the early diagnosis or target-treatment of fibrotic tissues. Similar probes targeting specific Fn conformation change have been reported, including phage-based molecular probes LNLPHG and RFSAFY (Cao *et al.*, 2012) and peptide probes FnBPA5 (Arnoldini *et al.*, 2017); however, further work need to be done to evaluate the specificity and limitation.

There are a number of challenges in this field including but not limited to the following:

1) Most approaches that have been developed remain at the *in vitro* and *ex vivo* levels, and it remains necessary to generate a harmless but convincing probe that can be used *in vivo*.

2) Resolution and sensitivities requires improvement. Force in the range of a few pNs can be detected in studies of actomyosin contractile force (Finer, Simmons and Spudich, 1994) and single strand DNA molecules (Bustamante, Bryant and Smith, 2003). However, the fN (femtoNewton) range might be the case referring to subcellular recording.

3) The techniques of probing the cellular force usually require complicated expertise to operate and produce, resulting in difficulties and high cost to be used as laboratory tools.

6.2 AmotL2: missing puzzle for vascular conundrum?

Transforming vein to artery?

Every year millions of patients with coronary atherosclerosis are operated on worldwide via heart and peripheral bypass surgery. Surgeons use the saphenous vein to replace the clogged coronary arteries due to easier access to the vein material. However, half of vein graphs fail after 10 years due to unexpected atherosclerosis (McKavanagh *et al.*, 2017). The sudden exposure to the increased shear stress and wall tension damaging the endothelium and SMC is one of the main causes of graft failure (ANGELINI *et al.*, 1987).

Abrogation of AmotL2 in adult mice is not lethal but affects actin filaments that connect the ECs to each other. This results in a cytoskeletal phenotype and shape similar to venous endothelium and raises the question of whether introducing AmotL2 into venous endothelium could confer stability to venous ECs subjected to higher flow. A well established mouse model of venous bypass graft arteriosclerosis with a perivascular "cuff" has been developed (Zou *et al.*, 1998), in which a vena cava piece from donor mouse is end-to-end grafted into carotid arteries. We may re-engineer the endothelium with AmotL2 by Lentvirus transfection before grafting and evaluate whether enhanced F-actin and junctional binding enable venous endothelium to sustain arterial flow.

Rescuing aging blood vessels?

According to data from various American cohort studies, about 60% of middle-aged men and slightly more than 50% of middle-aged women develop cardiovascular disease in their life (http://www.ehnheart.org/cvd-statistics.html). Apparently, aging is a significant risk factor for cardiovascular diseases. We noticed from our study that AmotL2 is expressed in arteries of mouse retina, but barely detected in 15-month-old mice. We therefore hypothesize that the ability of EC to sustain shear stress might be age-dependent. To investigate this, we analyzed AmotL2 expression level during mice aging (1-20 months) and found a negative correlation between AmotL2 and age in mouse lung (ECs account for 30%) and heart tissue (Stanford public database http://cmgm.stanford.edu/~kimlab/aging_mouse/).

Moreover, there is strong experimental and clinical evidence that "inflammaging" is a key contributors to aging (Franceschi *et al.*, 2000), and age-related vascular inflammation is a major cause of macro- and micro- vascular pathologies (Ungvari *et al.*, 2018). Considering that the absence of AmotL2 induce an inflammatory response, we might be able to inhibit vascular inflammation by restoring AmotL2 and thus subsequently arrest vascular aging.

6.3 YAP/AmotL2 signaling in vascular system and cancer

Previous report has confirmed AmotL2 as a direct transcriptional target of YAP from human MCF10A cells, mouse NIH3T3 cells and mouse liver tissue (Dupont *et al.*, 2011). In Paper IV, in an endothelial system, we provide strong evidence of a positive correlation ($r = 0.80$) of AmotL2 and YAP expression in the media of human aortae derived from AAA patients. This is of interest in evaluating whether a YAP signaling cascade includes AmotL2.

YAP has been identified as a major response factor of the mechanical force and implicated in vascular diseases due to its dysfunction (He *et al.*, 2018). However, its function cannot simply be concluded. The inhibition of YAP suppresses vascular inflammation and retards atherogenesis (Wang *et al.*, 2016c); however, YAP up-regulation acts as a protection factor for aortic dissection (Liu *et al.*, 2017). In addition to the vascular system, extensive studies have suggested that YAP is a tumor promoter in various cancer types, as reviewed by (Shibata, Ham and Hoque, 2018). An obvious challenge for the use of YAP as a therapeutic target is the question of how to balance the expression of YAP systematically and avoid the off-target effect. This motivates us to consider and research its downstream targets in specific physiological and pathological processes. After all, patients do not want to suffer from a new disease by the treatment.

To close my thesis:
Beyond the known unknown, there is always the unknown unknown to discover.

7 ACKNOWLEDGEMENTS

During my PhD journey in **Karolinska Institute**, I consider myself extremely fortunate to receive enormous support and generous help from my supervisors, co-workers, friends and family. I just cannot express my gratitude enough only by words in these pages.

My supervisors
My main supervisor Prof. **Lars Holmgren**, thank you for accepting me as your PhD student. It has been a delightful and precious experience for these years. We have built up a mutual understanding that we could get each other's point without words. Frankly speaking, the pursuit of this PhD is not an easy path. There were times I had doubts and frustration. However, you always believed me, with a warm smile and soft tone of voice, saying that I had been doing great and there would be success in the end waiting for me. There is an old saying in China: 授人以鱼不如授人以渔 (Give a man a fish and you feed him for a day; teach a man to fish and you feed him for a lifetime). This is exactly how you have been training me. I appreciate every time you listen to my thoughts, plans, and arguments, even though some of them are not mature. I am also grateful for the freedom you have granted me, so I could test and polish my own ideas independently. Thanks a lot for your patience, trust, support, and forever-positive attitude☺.

My co-supervisor Dr. **Yujuan Zheng**, with full understanding and patience, you introduced me to the group and the projects at the beginning. Thanks very much for teaching me important techniques and sharing me interesting thoughts.

Prof. **Chunyan Chen**, my supervisor during my master in Shandong University, you generously helped me with this opportunity to achieve my PhD degree in KI. Thanks a lot for your selfless support! I wish you and Prof. **Jihui Jia** the best.

Thank you my mentor Prof. **Joseph Bertrand**, your sincere comments once gave me lots of strength and confidence!

Key collaborators
Paper I, II and III. **Sumako**, it has been such a smooth and relaxing period for us to work together. Your self-discipline and motivation in research impressed me. I miss you! **Giusy**, without your generous effort and energy, we could not have completed these nice stories. You are also a sweet friend to me! **Yumeng**, thanks for being so kind and helpful with a self-giving heart. I appreciate all your contribution. Please keep the positive attitude that you have now, I am looking forward to your success! **John**, even you are in a different time zone in the US, you always give me the fastest response and help me fix problems. I am sure you will excel in your new career! **Nick**, thanks for all those inspiring discussions with me.
Big thanks to Prof. **Tom Barker** for providing me with constructive guidance and warm encouragement☺.

Paper IV. My dear **Evelyn**, you are one of the most organized people I have ever known, and you have been making your most effort for the projects without hesitation. You are the best collaborator I can wish to have! Good luck with everything in your new journey! **Sara**, I am so lucky to meet you in lab, for you have been so kind and helpful to me. I always feel safe and relaxed when you are around. **Maria Jose**, you impress me a lot with your high

efficiency and quality of your work! Thanks for doing endless qPCR for us. **Otto**, our discussions always open a new window for our story and big thanks for your effort!
Dr. **Daniel Ketelhuth** and Prof. **Per Eriksson**, thanks a lot for introducing comprehensive knowledge to me when I entered a new area. Special thanks to **Anna**, your excellent skills in IHC staining made my life much easier!

Other projects. **Aravindh**, it has been seven years since we worked together and you are more than a colleague to me! Thanks for your company and great memories with you, full of laughter, tear and craziness. Wish the best for your new career! Thanks **Amber**, for making such beautiful artistic graphs for my projects.

For those who has been leading me on the way up to academic pyramid.
Xiaolu Zhang, it has been 10 years since we met. You have been a trustful senior mate, a heart-warming sister, a lovely friend and a shining star for me. Thanks for all your support and guidance. **Xianli Shen**, "Nature man", thanks for being there, giving me support and sharing your experience. You are a role model to me! **Daria**, thanks for taking me to a night shift in hospital. That was such a cool experience. You are such a generous person sharing useful information and knowledge with me! **Na Wang**, you always offer your valuable opinions when I feel confused in both work and life.

My lab mates
Vicky, it has been such a happy time since you came to the lab. Thanks for always caring about me and supporting me. I truly appreciate the laughter and touching moments you have brought to me. Wish you a great experience of your PhD studies. **Pedro**, you are the most helpful people in lab. Thanks for sharing your scientific thoughts, project suggestions and positive attitude with me. Good luck with your projects. You deserve the best! **Aarren**, thanks for your generous help in proofreading. I am so impressed with your scientific passion. Keep fighting! **Seema** and **Honglei**, welcome to join the lab!

Phaboo, it is so enjoyable of those adventures we shared in Sweden, Switzerland, Czech, Thailand and China. Let's make more. **Stephanie**, those wonderful times we spent together always come back and warm my heart. **Katha**, I miss you for your enthusiasm in life and work. Keep fighting, young lady! **Yori**, I still cannot believe that we achieved that 8-week/30-minute running plan. Thanks for encouraging me to make the impossible possible! **Zhendong**, **Tanja**, **Tomas**, **Yuchen**, **Mahdi** and **Sebastian**, big thanks for those scientific discussions and fun time in lab.

My colleagues and friends
For those who have spent time with me in KI: **Lidi/Peng, Yuanjun**, and **Ran**/**Xinsong**, I am so grateful to have you around me, so we could discuss science, projects, and life. **Wei Han**, one of my oldest friends in Sweden, thanks for your generous support on some critical points over years. **Min**, what a cozy time we had for years. Soon it is your turn to graduate! **Muyi**, you are very talented. Keep fighting and you will be there! **Ishani**, you smile is like sunshine☺**. Mathilde**, **Dalel**, **Patricia**, and **Johanna**, thanks for the pleasant time in CCK. **Lina**, **Alessandro**, **Mercedes**, **Monika**, and **Pablo**, we have been lab neighbors from CCK to BioClinicum. Thanks a lot for daily talks and the emergent help. **Ziqing**, **Kristina** and **Tom**, our talks are so informative and inspiring. Great thanks for all of your company: **Qiang**, **Qing/Xiuming**, **Hongya**, **Xiaonan**, **Hao**, **Ninni**, **Yu Gao**, **Yujiao**, **Xiaotian**, **Xiangling**,

Ning, **Xia**, **Huaitao**, **Jiwei**, **Yaxuan**, **Dawei**, **Yingbo**, **Wen-Kuan**, **Elin**, **Yasmin**, **Sofia**, **Emarn, Makto**, **Neo**, and **Rong.**

Thanks for all the professional suggestions, comments, and warm encouragements from senior researchers: **Charlotte**, **Arne**, **Katja**, and **Rainer**. I'd like to express my sincere gratitude to administrators **Erika**, **Sören**, **Elisabeth**, **and Elle** in our department for fixing small-but-crucial problems for me. In addition, thanks to all the technicians who have spent loads of effort in maintaining our mice strains at MTC, AFL, KMW, and KMA.

With the time flying, I feel increasingly more attached to you☺.
Xuan, your presence is such a gift to me. I truly enjoyed every time we met for concert, shopping, and fika! Hope we can discover more beauty in life together. Good luck with your new adventure. **Limin**, my dear friend and neighbor, you have always been kind and positive to me. I cherish our lovely memories at work and off work. **Robert** and **Ylva**, It has been such a pleasure to know you. Thanks for your kind help and support, especially during those hard times. **Hong Su**, I will never forget those days we supported and comforted each other. We became much stronger together. Wish you the best with **Yongkun**. **Chen Qiu**, I wish we could have spent more time with each other. I miss you very much. Best wishes to you, **Ning Yang** and **Milan**.

Robin, **Anna**, **Jimmy**, **Cristofer**, **Nely G**, **Johan**, and **Yang,** thank you all for those fun time, which made my life more enjoyable. **肖文珍**, **赵乐康**, **李明**, **叶琳**, **顺哥**, **庆圆**, **李莎**, **仵霄**, and **郑琳**, even we are in different places in the world, your kind concern and support always keep me up to the best. **Jianlin**, 老铁, your encouragement got me through the tough periods. I admire you as a true traveller with your adventurous spirit! Thanks for sharing me new things around the world.

My love **Yan**, since we were 14, you have been the sunshine in my life. You always cheer for my accomplishment and show empathy when I feel sad. Besides the sweet girl talks, our arguments on philosophic topics made us grow together, regardless of 7252 kilometers in between. You have fulfilled all my imagination of a true friend. 感谢咱爸咱妈对我无条件的信心和鼓励，祝愿你的小家平安如意，静远猷远健康成长。

My family
My dear mom **Kemin Sun**, you have been an amazing woman in my eyes. You have not only achieved a fruitful career, but also kept learning new things after retirement. Wish you a great artwork exhibition soon! To my father **Xin Zhang**, a marine biology researcher, you have set me a solid example of how to be a scientist: always be serious in what you are doing and think twice before making judgement. Thanks you both for your unconditional love and positive influence on me. 我爱你们。

姥姥，谢谢您抚育我长大并教会我人生的道理，您的爱给予了我前进的力量。已远去的**姥爷**，**爷爷**和**奶奶**，你们都是有智慧的读书人，无论时代带来怎样的顺境与逆境，都无法掩饰你们的才华。希望你们看到我的努力。并为我而骄傲。感激所有家人对我的赞许和鼓励，你们是我的最强后盾。**黎黎**的小宝宝，姨妈迫不及待想见你啦!

My sister-in-law **Eva**, You have been such a caring person to me. You and **Robert** made me feel that I have family support in Sweden as well. Thanks to **Daniel** and **Caroline**, for your sweet parties and fun we had together!

To my husband **Olle**, the most important person during my PhD studies, you have strong belief in science and great passion for work. Your ungrudging support and genuine love lead me to move forward. Thanks for "pushing" me to fight for my scientific ambition and holding my hands through the difficult time. Jag älskar dig!

Grants/Fundings

As a student in **Karolinska Institute**, I feel so privileged to benefit from such resourceful education. Among those learning opportunities, I have attended and presented my research in six international conferences, which were kindly sponsored by **Radiumhemmets Forskningsfonder** and Prof. **Bengt Fadeel** (representing **STINT Grant)**.

Chinese Scholarship Council (CSC), thanks for granting me 4-year salary for my PhD study. The projects in this thesis were generously supported by grants received from **Swedish Cancer Society, Swedish Heart and Lung Foundation, Swedish Childhood Cancer Foundation, Cancer Society of Stockholm, Swedish Research Council**, and **Knut and Alice Wallenberg Foundation.**

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